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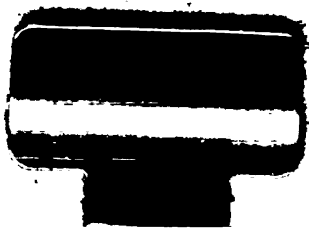
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CONTENTS

FURTHER STUDIES ON THE BEHAVIOR OF BACTERIA TOWARD GENTIAN VIOLET.*

BY JOHN W. CHURCHMAN, M.D.

(*From the Department of Bacteriology of Cornell University Medical College, New York.*)

PLATES 75 TO 77.

Isolation of a Gentian-Positive Strain from a Culture of a Gentian-Negative Organism (a "Strain-within-a-Strain" Variant).

When divided gentian violet plates are stroked with broth cultures or with heavy suspensions of *Bacillus coli*, growth is equally good on both sides of the plate (Fig. 1). So, too, if divided plates are poured so that each half receives a heavy inoculation of *Bacillus coli*, numerous colonies appear in both halves, though a similar experiment with *Bacillus subtilis* shows complete sterility of the gentian violet side. If, however, the inoculations are made with increasingly weak dilutions of the suspension, the colonies gradually become fewer on the gentian violet side and finally disappear altogether (Fig. 2). This phenomenon is exhibited whether a 2 or a 24 hour culture is studied. It may be demonstrated as well by a slightly different type of experiment. If a plain agar plate is stroked with broth cultures of *Bacillus coli* which have been exposed to the dye, the stained organisms appear to grow as well as the controls, though strokes of stained Gram-positive organisms, for example, *Bacillus subtilis* or *Oidium albicans*, will not grow at all (Fig. 3). If, however, the experiment is repeated with increasingly weak suspensions of *Bacillus coli* it will be seen that by no means all the organisms have actually survived exposure to the stain (Fig. 4).

* A preliminary report of this work was published in the *Proceedings of the Society of Experimental Biology and Medicine* (Churchman, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1920, xviii, 17, 19, 20, 21).

It is therefore possible, by stroking divided plates with weak dilutions of *Bacillus coli* to obtain plates, with a few colonies on the plain agar side and none on the gentian violet agar. Some of these colonies may be presumed to have sprung from individuals which would have grown on the gentian violet agar if they had happened to be implanted there, others may be presumed to be made up of organisms incapable of growing in the presence of the dye. By again stroking divided plates with suspensions of these separate colonies an opportunity was afforded of isolating a strain of *Bacillus coli* incapable of growing in the presence of the dye, unmixed with individuals capable of such growth. This has, indeed, been done.

The result of this experiment was to provide two strains—labelled for convenience Strains X and Y—isolated from a single "pure" culture of *Bacillus coli*. These two strains, both definitely Gram-negative, are identical by all the usual tinctorial, morphological, and cultural tests. Yet Strain X grows well, Strain Y not at all, or practically not at all in the presence of gentian violet (Fig. 5); and strokes of Strain X which have been exposed to the dye and then planted on plain agar grow equally well with the controls, while strokes of Strain Y made with organisms which have been exposed to the dye do not grow at all (Fig. 6).

That this specific characteristic is not transient is shown by the experiment represented in Figs. 5 and 6. This experiment was done after the two strains had been allowed to grow for 6 weeks through a number of transplants, and it will be seen that the original features of the two strains have been retained.

The experiments outlined above demonstrate that within a single bacterial strain two strains may exist which, though identical in morphology and in tinctorial and cultural reactions, are dissimilar in their reactions to gentian violet, one growing vigorously and the other not at all on media containing the dye. These two types retained their differential characteristics after many transplantations. This isolation, from a pure culture, of a "strain-within-a-strain" variant parallels the observation reported some years ago of a "strain-within-a-species" variant occurring in the *Bacillus enteritidis* group.[1]

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[1] Churchman, J. W., and Michael, W. H., *J. Exp. Med.*, 1912, xvi, 822.

The distinction between the two types of variant strain is, of course, not fundamental; one type was encountered in nature, the other was isolated in the laboratory.

Explanation of the Parallelism between the Gram Reaction and the Gentian Violet Reaction.

While certain facts in the observations hitherto reported on the parallelism between the Gram reaction and the gentian violet reaction were difficult to explain on the theory that the two reactions depended on a common factor and that the parallelism was therefore a fundamental one, the preponderance of evidence pointed in this direction. About 90 per cent of the Gram-positive organisms are killed by exposure to the dye and will not grow on media which contain it; about 90 per cent of the Gram-negative organisms grow well after exposure to the dye, and their growth seems to be unimpeded by the presence of the dye in media.

That this difference between the effect of gentian violet on the Gram-positive and the Gram-negative organisms might be applied to the isolation of Gram-negative bacteria from mixtures with Gram-positive bacteria, and particularly to the elimination of contaminations with *Bacillus subtilis*, which so frequently prove troublesome, was pointed out in a previous publication.[2] The lead thus given has since received the following practical applications: (1) isolation of *Bacillus tuberculosis* from sputum and feces (Petroff[3]); (2) elimination of false presumptive tests in the examination of milk and water for *Bacillus coli*, by the use of gentian violet (Hall and Ellefson[4]); (3) selective elimination of hay bacillus from cultures of obligative anaerobes by the use of gentian violet (Hall[5]); (4) isolation of *Bacillus influenzæ* by the use of gentian violet (Bernstein and Loewe[6]); and (5) isolation of pathogenic moulds (*Epidermophyton inguinale*) by the use of gentian violet (Farley[7]).

[2] Churchman, J. W., *J. Exp. Med.*, 1912, xvi, 221.

[3] Petroff, S. A., *J. Exp. Med.*, 1915, xxi, 38.

[4] Hall, I. C., and Ellefson, L. J., *J. Bact.*, 1918, iii, 329, 355; *J. Am. Water Works Assn.*, 1919, vi, 67.

[5] Hall, I. C., *J. Am. Med. Assn.*, 1919, lxxii, 247.

[6] Bernstein, E. P., and Loewe, L., *J. Infect. Dis.*, 1919, xxiv, 78.

[7] Farley, D. L., *Arch. Dermatol. and Syphilol.*, 1920, ii, 466.

A parallelism so clear-cut and so amply confirmed could hardly be accidental, particularly as a similar parallelism was found to exist if the experiments were conducted with other dyes belonging to the triphenylmethane series. Moreover, it is just the constantly Gram-positive organisms like *Bacillus subtilis* which always fail to grow in the presence of even minute quantities of the dye, and just the constantly Gram-negative ones which grow readily in fairly strong dilutions of it (Fig. 1).[8]

The difference in avidity of the living organisms for the stain is also striking. *Bacillus subtilis* rapidly becomes deep violet and loses its motility; *Bacillus coli* or *Bacillus typhosus* stains slowly, only a small proportion of the individuals stain at all deeply, and all retain their motility for some time. If the Gram reaction is carried out with living organisms, centrifuged in a test-tube, the behavior of the negative and positive groups is the same as it is when they are killed and stained on a glass slide.

Facts of this kind led to the assumption that the parallelism between Gram reaction and gentian violet reaction depended on the presence in the Gram-positive organisms, and the absence from the Gram-negative ones, of unsaturated bodies avid for some portion of the triphenylmethane molecule. The saturation of these bodies would be thought of as accounting for the retention of the dye by the Gram-positive bacteria and their death in its presence; the absence of these bodies would explain why Gram-negative organisms fail to retain the dye and grow in media containing it.

There were, indeed, a few facts which would not conform to this hypothesis. There were in the first place the exceptional organisms; about 10 per cent of the Gram-positive strains studied were either unaffected by the dye or variable in their reactions to it, and an equal percentage of the Gram-negative cultures were somewhat susceptible to its bacteriostatic effects. Not only was this found to be the case in a study of all the common bacterial species, but an instance

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[8] The chemical problems involved in the gentian violet reaction are now the subject of investigations in collaboration with Professor Bogert of Columbia University. It may be stated, however, that other members of the triphenylmethane series, which are themselves not dyes and possess relatively simple structural formulas, behave, as regards selective bacteriostasis, like gentian violet.

was encountered in which one strain of a species (*Bacillus enteritidis*) —and only one out of five studied—was quite sensitive to the bacteriostatic action of the dye, though the other four behaved quite according to rule, and all five were identical in cultural reactions and were definitely Gram-negative.[9]

An effort was made to test the fundamental nature of the parallelism between Gram and gentian reactions by training a Gram-positive and gentian-positive organism (*Bacillus subtilis*) to grow in the presence of the dye. If such an organism could be made in this way to lose its gentian positiveness without changing its Gram positiveness it would be clear that the two reactions did not depend on a common factor. If, on the other hand, both reactions changed, the opposite would be true.

The efforts to train *Bacillus subtilis* to grow in gentian violet were wholly unsuccessful. This organism will not grow on divided plates containing the dye in strengths of 1:1,000,000. It is possible, it is true, to induce it to grow in slightly stronger dilutions. But not much advance can be made, and the advance made is not retained; for the few organisms which have appeared in the stronger dilutions, if transplanted, fail to grow except in the weaker dilutions, around 1:1,000,000.

The problem was, however, solved in an entirely different way. If thick suspensions of the Gram-negative and gentian-negative *Bacillus coli* are stroked across a divided gentian violet plate, growth is equally vigorous on the two sides (Fig. 1). If, however, increasingly weak dilutions of the suspension are used for the stroking, the colonies on the gentian violet side of the plate become rapidly fewer (in proportion to those on the plain agar) as the dilution increases, and finally disappear altogether (Fig. 2). This is due, in part at least, to the fact, entirely obscured when thick suspensions are used for the experiment, that only a relatively small proportion of the

[9] It is interesting to note that Bronfenbrenner and his coworkers (Bronfenbrenner, J., Schlesinger, M. J., and Soletsky, D., *J. Bact.*, 1920, v, 24) in a study of the bacterial action of the CR indicator (China blue-rosolic acid) tested out this strain of *B. enteritidis* and found it to be exceptional also in its behavior toward CR. I have found that rosolic acid possesses selective properties similar to those of gentian violet.

individuals in a suspension of a Gram-negative organism are really gentian-negative; the majority will not grow in the presence of the dye.[10] If the suspension used for stroking the plate is thick, this small proportion of gentian-negative individuals is (absolutely) sufficiently large in quantity to produce good growth in the presence of the dye. If, on the other hand, the suspension is weak the gentian-negative individuals are, not only relatively, but absolutely few in number; few colonies therefore appear on the violet agar; if the dilution is very weak, none appears.

It is not difficult to isolate in pure culture the individuals which will and those which will not grow in the presence of the dye (Figs. 5 and 6). When so isolated the gentian-positive strain, that is the strain which will not grow in the presence of the dye, is found to be as definitely Gram-negative as the gentian-negative strain. The factor which determines the reaction of an organism to the Gram process of staining is not therefore the same as the factor which determines its growth reaction in the presence of gentian violet.

Effect of Repeated Reinoculations of Gentian Violet Agar with a Gentian-Positive Organism.

If a divided gentian violet plate containing the dye in a dilution of 1:100,000 is stroked with a thick suspension of the Gram-positive and gentian-positive *Bacillus subtilis*, no growth will ever occur on the gentian violet side of the plate; the organism will, indeed, fail to grow up to the dividing line between plain and gentian violet agar, ceasing sharply at a point 0.5 to 1 cm. from this line (Fig. 1). This inhibition takes place even when the dye is present in extremely weak dilutions. Growth is prevented by dilutions of 1:1,000,000 (Fig. 7), inhibited by 1:2,000,000, and only becomes vigorous at a strength of 1:3,000,000.

If, however, the gentian violet half of the plate is repeatedly and heavily reinoculated on successive days in the same place, a fair growth,—in some instances a rather vigorous growth—may finally

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[10] It cannot be said, since the whole bacterial field has not been studied with this point in view, that all Gram-negative species contain these two strains. The specimen of *B. coli* with which the experiments were done and the specimen of *B. typhosus* and *B. prodigiosus* examined contained them.

be obtained. In Fig. 8 such an experiment is shown. Two strokes of a thick suspension of *Bacillus subtilis* were made on this plate. Nothing further was done to the right-hand stroke and the usual effect of the dye is clearly shown. In the left-hand stroke from the edge where the original growth on the plain agar ceased (this edge will be readily recognized), organisms were repeatedly resmeared over the gentian violet agar; a fair growth resulted.

That this growth which results from restroking is not due to acclimatization of organisms to the dye seems likely from the fact that prolonged attempts at experimental acclimatization of *Bacillus subtilis* to gentian violet have been unsuccessful. Furthermore, positive proof that acclimatization is not the explanation is given by the fact that if organisms which have, by reinoculation, been thus induced to grow on gentian violet agar, are freshly smeared on another divided plate they will not grow on the side of the plate which contains the dye. This phenomenon can hardly be due to insufficiency of dye in the presence of increase of inoculated organisms, for in the plates used in the experiments dye was present in strengths (1: 100,000) greatly in excess of the strength necessary to prevent the growth of *Bacillus subtilis* (1:1,000,000); and owing to the great diffusibility of the dye through agar much of that present in the plates is available for the organisms.

Whether the phenomenon is due to a change in the dye caused by organisms which, though not surviving, live sufficiently long to prepare the soil for subsequent implants; whether it is due to some protective barrier or nutritional or growth-accelerating substance provided by the dead bodies of bacteria to the living bacteria which rest upon them in subsequent seedings; or whether it is due to a communal activity of organisms—to which further reference is made in the following paper—are questions at present under experimental study. But the fact itself is sufficiently significant for certain problems in the chemotherapy of infections.

In searching for a possible explanation for these facts it is important that attention should be called to experiments which demonstrate the effect of the presence of dead bacterial bodies in media on which transplants are made. An experiment of this sort is illustrated in Figs. 9 and 10. The tubes contained gentian violet agar.

The surface of the control tube (Fig. 9) was inoculated with *Bacillus subtilis* and, as this organism never grows in the presence of the dye, no growth occurred. The surface of the other tube (Fig. 10) was covered with a thin layer of killed, washed organisms[11] (*Bacillus subtilis*). After this layer dried an inoculation of living *Bacillus subtilis* was made on top of it. Growth occurred, owing apparently to a complete protection of some sort interposed by the dead bodies between the living bacteria and the dye, or to some effect of the dead bacteria on bacterial growth. A similar result is obtained if dead bodies of *Micrococcus aureus* or of *Bacillus coli* are smeared on the surface of the gentian violet agar instead of dead bodies of *Bacillus subtilis*. Such an experiment, besides bringing out a fact to which attention has not hitherto been directed, shows clearly that dead bacterial bodies lying in a wound may entirely alter the reaction between a bacteriostatic agent and the living organisms which one is attempting to reach.

Selective Activity of Gentian Violet in Relation to Chemotherapy.

That the selective bacteriostatic action of gentian violet, demonstrable *in vitro*, is capable of clinical application in the treatment of infections has been amply demonstrated by experiments conducted in the hospital wards during the last 6 years. It has been shown that in certain types of acute infections of joints, staining of the synovial membrane (after proper mechanical cleansing by special methods) leads to prompt sterilization and clinical cure.[12] It has also been shown that with this dye it is possible to rid granulating wounds of

[11] The organisms were grown on medium containing only distilled water and agar, in order that their bodies might contain the minimum amount of nutritive substance. These bacterial bodies were washed six times with distilled water and were killed. Suitable controls were made to insure sterility. In order to prove that no available food material was still present, a control experiment was done in which these dead bacterial bodies were smeared on the bottom of a Petri dish, dried, and then inoculated with live *B. subtilis*, suitable moisture being provided. No growth occurred on incubation. The growth of *B. subtilis* in the experimental tubes illustrated in Fig. 10 was not, therefore, due to any nutritive material carried over in the dead bacterial bodies.

[12] Churchman, J. W., *J. Am. Med. Assn.*, 1918, lxx, 1047; 1919, lxxii, 1280; 1920, lxxv, 583.

certain organisms which have resisted other bacteriostatic agents; this was proved by the successful treatment of two patients with thigh amputations at the Walter Reed Hospital. The stumps of these individuals had become diphtheria carriers, but by using the dye they were rid of organisms which had persisted for weeks in spite of all treatment.[13] That the selective power of the dye, so clearly shown in divided plates, would also be demonstrated by a study of its effects in wounds was well shown during these efforts to sterilize infected amputation wounds. *Bacillus diphtheriæ* was caused to disappear without much difficulty, but in a stump infected with *Bacillus coli* it was impossible to rid the wound entirely of its organisms.

In spite of this considerable amount of clinical success in the treatment of a certain type of infection with gentian violet, the observations reported in the previous communications indicate that selective bacteriostasis is a very complex process and point out the difficulties attending the direct transfer of laboratory findings into therapeutics. The method of divided plates presents an excellent means of studying the interaction between dye and organism; the isolation of a pure strain of gentian-negative *Bacillus coli* makes it possible to investigate the question by single cell experiments of a peculiarly reliable kind; and the selective property of gentian violet may be taken advantage of for the study of the various elements of selective bacteriostasis on a single plate. Such a plate is represented in Fig. 11. This experiment was done to demonstrate the futility of generalizations about the effect of a chemotherapeutic agent on bacteria, even when the generalization is confined to its effect *in vitro*, and to indicate the necessity of bearing in mind, when drawing conclusions from experiments in this field, the conditions under which the experiment was done. On this plate, as will be seen (Fig. 11) seven different effects of gentian-violet on bacteria are shown, the result obtained depending on the organism chosen for the experiment and the conditions under which the inoculation was made. These different results are as follows: (1) Thick suspensions of Gram-positive organisms (*Bacillus subtilis*) will not grow in the presence of the dye (Fig. 11, *A*). (2) By repeated reinoculations of these organisms a moderate

[13] Churchman, J. W., *J. Am. Med. Assn.*, 1920, lxxiv, 145.

growth can be procured on the gentian violet agar (Fig. 11, *B*). (3) Thick suspensions of the Gram-negative *Bacillus coli* grow equally well on the two halves of the plate (Fig. 11, *C*). (4) If weak dilutions of a suspension of *Bacillus coli* are stroked across the plate almost no growth occurs on the gentian violet side (Fig. 11, *D*). (5) A thick suspension of the gentian-negative strain of *Bacillus coli* grows equally well on the two halves of the plate (Fig. 11, *E*). (6) If a weak dilution of the gentian-negative strain of *Bacillus coli* is stroked across the plate no growth may occur on the gentian violet side (Fig. 11, *F*). (7) If a thick suspension of the gentian-positive strain of *Bacillus coli* is stroked no growth occurs on the gentian violet agar (Fig. 11, *G*).

Unless considerations of this kind are borne in mind one may easily fall into gross error. For example, if the original experiments had been conducted with a thin suspension of *Bacillus coli* and the result seen at *D*, Fig. 11, had been obtained the conclusion would have been drawn that gentian violet has a strong bacteriostatic effect on *Bacillus coli;* whereas if thick suspensions are used, the opposite proves to be the case (Fig. 11, *C*). This error has, as a matter of fact, been committed by certain observers working with other dyes.

Since the single cell method of Barber[14] offers an important method for the study of reactions between chemical substances and single bacteria, it cannot be too strongly emphasized that the effect of a bacteriostatic agent on a group of bacteria may be quite different from its effect on individual organisms.

That the effect of a chemotherapeutic agent should be conceived in terms of stasis rather than death has been proved by the whole trend of the work with gentian violet. The difficulty of proving that organisms which have apparently been killed by chemical agents are really dead may be greater than might at first be supposed; and in any experiments in this field it is well to remember that organisms apparently killed may survive and give signs of life after a long period of lag. A striking instance of this phenomenon is shown in Text-fig. 1. This experiment was one of a series done to determine by animal inoculations whether stained gentian-positive organisms, which do

[14] Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

not grow in culture media, are actually killed by exposure to the dye. Two series of animal inoculations—one with *Staphylococcus aureus* and the other with a strain of *Blastomyces* pathogenic for guinea pigs —seemed to warrant the conclusion that the stained organisms were killed, for all the animals that received unstained organisms died of the disease, and all those that received stained organisms survived. With stained *Bacillus anthracis*, however, different and striking results were obtained.   This organism will not grow when it is planted on agar after having been stained.   When injected in large amounts,

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
M.1	—	X																										
M.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	X		
M.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R.1	—	—	—	—	—	X																						
R.2	-	-	-	-	-	-	X																					
R.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TEXT-FIG. 1. Results of animal inoculation with stained *B. anthracis*. *M.*, mouse; *R.*, rabbit.   The course of control experiments is represented by a solid line, the course of experimental animals by a broken line.   *X* indicates that cultures made at death from the heart's blood and spleen were positive for *B. anthracis*.   Mouse 3 and Rabbits 3 and 4 survived without symptoms.   Mouse 2 died of anthrax on the 26th day; the blood and spleen swarmed with the organisms.

after staining, into the susceptible mouse, it often causes death, though not infrequently the inoculated animal lives.   But the point worthy of note is that when the animal dies it is sometimes after a period of many days of perfect health, though the control mice die in 36 hours.   Mouse 2, for example (Text-fig. 1) died of anthrax on the 26th day, having been in perfect health in the interval; that is, it behaved as if it had received the injection more than 3 weeks after the inoculation had actually been made.   The stained organisms, nearly but not quite killed by their exposure to the dye, had suffi-

ciently recuperated at the end of 26 days to bring about the death of the animal from anthrax. In the animals which survived the inoculation entirely it is not unlikely that the defensive mechanism of the body was successful in overcoming the bacteria, which were injured but not killed. The experiments prove that the survival of injected animals does not necessarily prove that the organisms injected were dead.

SUMMARY.

1. A gentian-positive strain (a "strain-within-a-strain" variant) has been isolated from a pure culture of a gentian-negative organism. This observation corresponds to that of a "strain-within-a-species" variant, occurring in the *enteritidis* group, reported some years ago.

2. The Gram reaction and the gentian reaction do not depend, as has been assumed in previous publications, on the specific affinity of the gentian-positive organisms for a portion of the gentian violet molecule, since certain Gram-negative strains are shown to be gentian-positive.

3. Dead bacterial bodies interposed between living bacteria and gentian violet media partially negative the effect of the dye on Gram-positive organisms and allow them to grow. This seems to be either a phenomenon of filtration or of stimulation of growth.

4. The application of these facts by the method of divided plates shows a number of difficulties in the application of laboratory studies to chemotherapeutics, which would escape observation by ordinary methods.

EXPLANATION OF PLATES.

PLATE 75.

Fig. 1. Divided gentian violet plate (gentian violet 1:100,000). *T, B. typhosus; S, Micrococcus aureus; OA, Oidium albicans; Su, B. subtilis; C, B. coli.* It is the strongly Gram-positive organisms that fail to grow and the strongly Gram-negative ones that grow.

Fig. 2. 24 hour cultures of *B. coli*. Disappearance of all colonies from the gentian violet agar side of the plate as the dilution of suspension used for stroking becomes weaker. *C, B. coli; Broth*, broth culture (undiluted).

FIG. 1.

FIG. 2, *a.*

FIG. 2, *b.*

FIG. 2, *c.*

(Churchman: Behavior of bacteria toward gentian violet.)

FIG. 3.
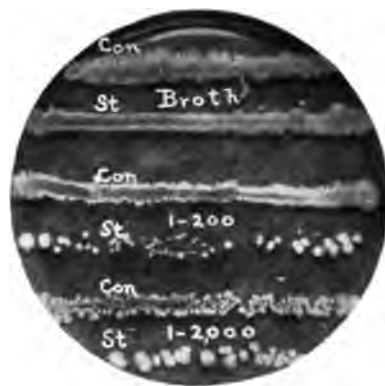
FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

FIG. 8.

(Churchman: Behavior of bacteria toward gentian violet.)

PLATE 76.

FIG. 3. Effect of staining on Gram-negative *B. coli* and Gram-positive *Oidium albicans* (*cf.* Fig. 4). *C*, unstained *B. coli* (control); *C'*, stained *B. coli; O*, unstained *Oidium albicans* (control); *O'*, stained *Oidium albicans*.

FIG. 4. Effect of staining increasingly weak dilutions of a suspension of *B. coli* with gentian violet. *Con*, unstained organisms; *St*, stained organisms.

FIG. 5. Behavior of Strains X and Y on a divided plate 6 weeks after first isolation.

FIG. 6. Behavior of Strains X and Y on exposure to gentian violet 6 weeks after first isolation. Plain agar plate. *X*, unstained organisms (control); *X'*, stained organisms. *Y*, unstained organisms (control); *Y'*, stained organisms.

FIG. 7. *B. subtilis* on a divided plate. The upper half contains gentian violet in 1:1,000,000 dilution. (*A* refers to the series of experiments.)

FIG. 8. Two strokes of a thick suspension of *B. subtilis* were made. On the right nothing further was done. On the left reinoculations were made on the gentian violet agar.

PLATE 77.

FIG. 9. Implantation of *B. subtilis* on the surface of gentian violet agar; no growth resulted. *a*, site of inoculation.

FIG. 10. Implantation of *B. subtilis* on gentian violet agar the surface of which had been covered with a thin layer of killed, washed organisms. *a*, colony of *B. subtilis; b*, layer of killed washed organisms (*B. subtilis*); *c*, photograph of the surface of the tube, showing the colony of *B. subtilis*.

FIG. 11. The figure shows how the result of an experiment in glass on the effect of a bacteriostatic agent may vary with slightly changing conditions and illustrates some of the difficulties encountered. *A*, stroke of a suspension of *B. subtilis; B*, stroke of a suspension of *B. subtilis* with reinoculation of gentian violet agar; *C*, stroke of a thick suspension of *B. coli; D*, stroke of a thin suspension of *B. coli; E*, stroke of a thick suspension of Strain X; *F*, stroke of a thin suspension of Strain X; *G*, stroke of a thick suspension of Strain Y.

COMMUNAL ACTIVITY OF BACTERIA.*

BY JOHN W. CHURCHMAN, M.D., AND MORTON C. KAHN.

(*From the Department of Hygiene of Cornell University Medical College, New York.*)

PLATES 78 TO 80.

The isolation of a strain of *Bacillus coli* fast to gentian violet, that is one containing no individuals susceptible to the bacteriostatic properties of the dye,[1] has made it possible to study quantitatively the reaction between this bacteriostatic agent and bacteria, without encountering the disturbing factor usually met in such studies which results from the variability in susceptibility of individual organisms to the chemical substance under examination. This strain —Strain X—had been isolated from a single colony growing on gentian violet agar, and had been kept growing, by frequent transplants, on media containing the dye, over a period of several weeks.[2] The ability of every individual to grow in the presence of the dye had therefore been proved.

With this strain a large number of single cell and small group transplants have been made in order to see whether any difference could be observed between the behavior of isolated individual organisms and that of very small aggregations of the same organisms. The experiments showed that a marked difference exists.

The technique used was that of Barber.[3] Only motile organisms were transplanted. The transplants were always made with young broth cultures 2 to 4 hours old, but it was found equally important to use for the stock cultures, from which the broth cultures for study were made, fresh agar transplants not more than 18 hours old; when

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* A preliminary report of these observations was published in the *Proceedings of the Society of Experimental Biology and Medicine* (Churchman, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1920, xviii, 22).

[1] Churchman, J. W., *J. Exp. Med.*, 1921, xxxiii, 569.

[2] See Figs. 5 and 6, of the preceding paper.[1]

[3] Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

older agar transplants were used there were delay and inconstant results in the controls. We observed this type of lag, due to the age of the culture from which the subculture for the transplantation was made, independently, before knowing of Chesney's work.[4] To bear it in mind in single cell work is essential.

To determine the behavior of single cells in the presence of gentian violet, transplantations were made onto gentian violet agar and into gentian violet broth, and by way of comparison, similar transplants of small groups of cells were made into the same media.

Transplantation onto Gentian Violet Agar.

Although, as has been said, the organism with which the experiments were done was definitely gentian-negative and grew with apparently no inhibition when strokes of a heavy suspension were made onto agar containing the dye,[5] transplants of single cells almost never grew, although as high as 85 per cent positives were obtained in the controls. In the only two instances in the whole series of single cell transplants in which growth occurred, marked delay took place, a delay which was never observed in the controls. Moreover, transplants of small groups of organisms (five to fifteen) did not grow, though transplants of thirty individuals grew regularly (Figs. 1 to 5).

Transplantation into Gentian Violet Broth.

The experiments just cited made it seem likely that there was some fundamental difference between the behavior of a single cell and that of a small group of cells in the presence of gentian violet and this probability became a certainty as a result of the transplantations made into gentian violet broth in a dilution of 1: 100,000, pH 7.2. The gentian negativeness of the organism used for this series of experiments was even more authentic than that of the one used in the agar experiments. It came from the colony marked B in Fig. 5. This was one of the only two colonies which ever appeared in our experiments after transplantation of a single cell onto gentian violet agar. Colony A on this plate was just visible at the end of 18 hours, though

[4] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.
[5] See Fig. 5 of the preceding paper.[1]

the controls were well developed at this time (Fig. 3). At the site where Colony B subsequently developed nothing was at this time to be seen, even with a magnifying glass; Colony B appeared during the second 24 hours.

The organisms in Colony B provided material for a study of the effect of gentian violet on single cell transplants that was above criticism. Not only had it come from a pure gentian-negative strain and been kept growing for weeks on gentian violet media, but the organisms in Colony B were all the descendants of a single cell which had survived and reproduced in the presence of the dye. One could feel certain that every individual in this culture was gentian violet-negative; one did not have to consider the possibility that failure of growth in single cell transplants might be a matter of chance and due to the presence of individuals susceptible to the dye, which happened to be picked up. As a matter of fact, this possibility of a chance picking up of susceptible organisms could hardly be seriously considered as an explanation of the results, if the large number of transplants made is borne in mind.

The pedigree of Colony B is shown in Text-fig. 1. The results of the inoculation of broth with this strain which was called Strain Z showed beyond doubt that there is a fundamental difference between the behavior of one cell and that of a small group of cells toward gentian violet. 80 per cent positives were obtained in the controls in which single cells were inoculated into plain broth; and almost 100 per cent positives were obtained when thirty or more cells were planted in gentian violet broth. Yet when single cells were planted in gentian violet broth, or when very small groups (two to eight organisms) were seeded, growth did not occur (Figs. 6 and 7). On the basis of 140 successive single cell transplants into gentian violet broth without growth in any instance, the conclusion seemed justified that single cells would never grow under these conditions. In a final series, however, of eight inoculations delayed growth was obtained in one tube in the second 24 hours, an occurrence so rare that it seems justifiable to regard it as the occasional and unexplained exception met with in the study of almost every biological phenomenon. These negative results with single cells were striking when compared with the almost absolute constancy with which growth occurred when groups of thirty or more cells were inoculated.

The term communal activity of bacteria is used to express the facts just detailed. This term is open to the objection that bacterial interreactions are implied, for which there is at present no rigorous evidence. It might be that thirty cells succeed in growing, merely because thirty cells are able to produce some antidye substance in an amount sufficient to destroy the bacteriostatic effect of the dye, while one cell fails to grow because it is unable to do this. Without
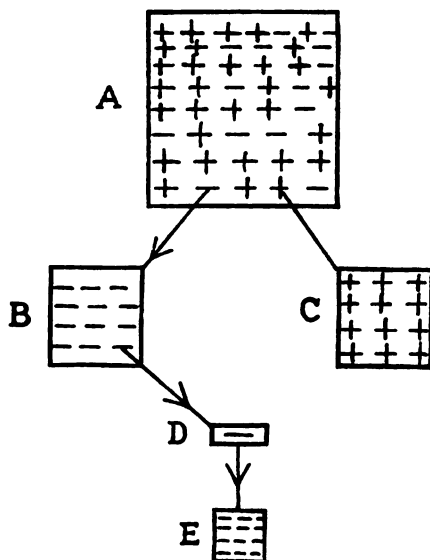
TEXT-FIG. 1. Pedigree of Strain Z. *A*, original culture of *B. coli*, containing gentian-negative and gentian-positive individuals; *B*, pure culture of gentian-negative strain, isolated from *A*; *C*, pure culture of gentian-positive strain isolated from *A*; *D*, single cell from Strain B; *E*, colony resulting from planting Strain D on agar.

implying anything as to the underlying source of the phenomenon we use the term communal activity to describe it in order to indicate that in the contest between bacteria and bacteriostatic agents very small groups of organisms may be able to accomplish together what they could not accomplish if working singly. The significance of these facts for the study of chemotherapy and particularly for the study of the effect of bacteriostatic agents by the single cell method is clear. One cannot conclude from the behavior of a single cell in the

presence of such a substance anything as to the behavior of a group of cells, even a very small group, under the same conditions.

It may perhaps be again emphasized that the results obtained in these experiments were clearly not due to individual variants, susceptible to gentian violet, which happened to be picked up in making the single cell transplants. Not only was the strain used one which had proved its insusceptibility to gentian violet by growing in the presence of this dye, but the failure of growth in 140 consecutive single cell transplants into gentian violet broth eliminates, by the law of probabilities, such an explanation.

It should be clear that the fact here established is of a different order from the well known relation between growth and gross size of inoculum. The presence of large numbers of dead organisms in the ordinary culture and of organisms which, though living, are susceptible to the slightly unfavorable conditions of the new media into which they are transplanted makes it more probable that growth will follow the inoculation of media with 500,000,000 organisms than if only 500 are used. But in the experiments described here only living, motile organisms were used, and only individuals from a strain which had proved its ability to grow in the presence of the dye and did so constantly when inoculated in groups of thirty or more. It seems probable that some factor not hitherto recognized must be found to account for the difference in behavior between one cell and thirty cells and that it is a factor other than that which accounts for the difference in behavior between 500 cells and 500,000,000.

We have done a large number of experiments to determine whether the facts just detailed were to be explained simply by the relation of the number of transplanted organisms to the amount of available gentian violet; that is, whether the whole phenomenon is a purely quantitative one. Large inoculations of this gentian-negative strain grow in the presence of gentian violet without any apparent restraint; so, too, do inoculations of thirty cells; whereas single cells, under identical conditions, do not grow at all. This might be due to the fact that groups of cells, even small groups of thirty individuals, were able to make some change in the dye, gentian violet being assumed to offer a slightly unfavorable medium even for this gentian-negative strain, in spite of the absence of any apparent inhibition to the growth

of groups of cells. Single cells might be unable to effect this change in dye in an amount sufficient to allow growth to take place. If this explanation were the correct one, it should be possible to demonstrate an approximate relation between the number of organisms and the amount of gentian violet broth in which a given group could grow. In the experiments thus far described the transplants were made into 5 cc. of 1: 100,000 gentian violet broth. Under these conditions one cell would not grow but thirty cells would. If it is assumed that this was because thirty cells could produce thirty times the amount of antidye substance produced by one cell, then by merely multiplying by thirty the amount of gentian violet broth into which the inoculations were made, that is by using for example 150 cc., the growth of thirty organisms should be prevented. As a matter of fact thirty organisms will grow, with a fair degree of constancy, not only in this amount of gentian violet broth, but in very much larger amounts. When inoculations of thirty organisms were made, for example, into a liter of gentian violet broth, growth occurred in 60 per cent of the flasks, though if the explanation of the phenomena described were purely quantitative it should be necessary to seed 6,000 organisms into this amount of gentian violet broth in order to obtain growth; that is, thirty organisms accomplish not thirty times what one organism accomplishes, but very much more than this. This discrepancy between the work actually accomplished by thirty organisms, and the work which they might be expected to accomplish on a purely quantitative basis is represented by the line $A-B$ in Text-fig. 2.

It might be objected that bacteria ought not to be·thought of as units exactly equal to one another in efficiency. A given group of thirty might be able to do only fifteen times the work of one very vigorous organism, while another group of thirty might be able to do 60 times the work of a relatively weak organism. If, however, there was any very marked individual variation in the ability of individual organisms to cope with gentian violet, this would have shown itself in occasional growths of vigorous cells when single cell transplants were made. Furthermore, such a variation would hardly explain why thirty cells can grow in a liter of gentian violet broth, and thus do what not less than 6,000 might be expected to do.
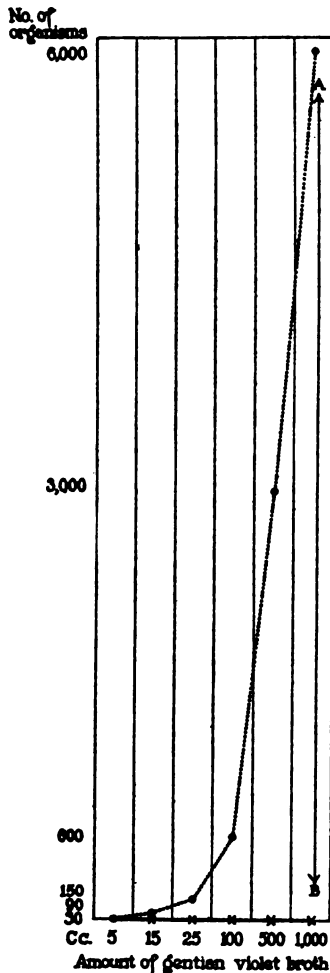
TEXT-FIG. 2. The solid line at the bottom of the chart represents the curve of growth of thirty organisms. These grow even in a liter of gentian violet broth. The dotted line represents the curve that would be expected if the growth of groups of organisms depended entirely on the quantity of available gentian violet; in a liter of gentian violet broth 6,000 organisms would have to be seeded to produce growth. The line A–B represents the discrepancy between what actually occurs and what would be expected to occur from a quantitative theory of the difference between the behavior of one cell and that of a group of cells.

That the amount of available gentian violet does, within very narrow limits, play some part in the prevention of growth of single cells is shown in Text-fig. 3. The dotted line A–B represents the results of single cell inoculations into gentian violet broth. It will be seen that single cells never grow in more than 0.8 cc., but that in amounts smaller than this a fair number of positives occur. When 0.1 cc. was used 60 per cent positives were obtained. The corresponding amount of gentian violet broth for thirty organisms would
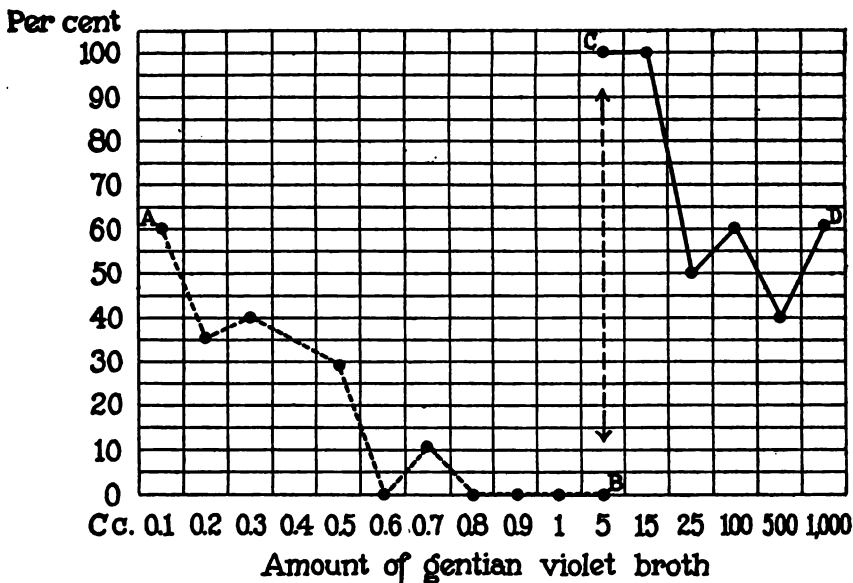
TEXT-FIG. 3. *A–B* is the curve of growth for single cells—0 per cent when 5 cc. of broth were used, 60 per cent when 0.1 cc. was used. *C–D* is the curve of growth for thirty cells, 100 per cent when 5 cc. of broth were used, 60 per cent when 1 liter was used.

be thirty times 0.1 cc., or 3 cc.; yet as a matter of fact 60 per cent positives were obtained with thirty organisms in over 300 times this amount of gentian violet broth; that is, in a liter (Text-fig. 3, line *C–D*). It is important to observe that the amount of broth used for the experiments, independent of the amount of gentian violet, did not play a part in the results, for in experiments made to determine this point, 75 per cent positives were obtained when single cells were inoculated into a liter of plain broth.
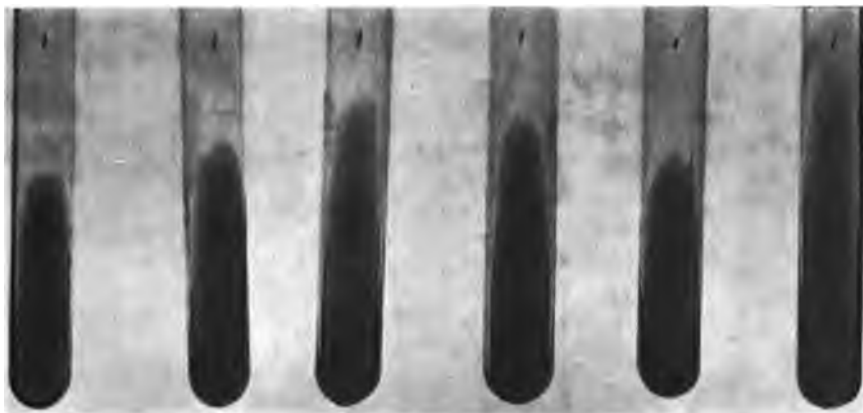
FIG. 1.
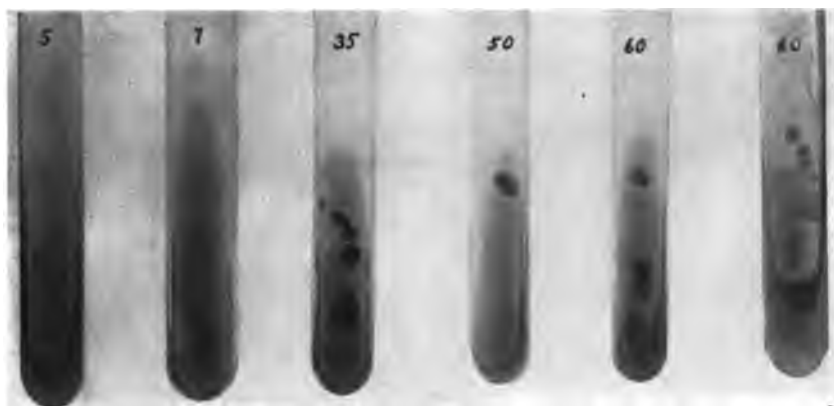
FIG. 2.

(Churchman and Kahn: Communal activity of bacteria.)

It seems clear, therefore, that thirty cells, instead of being able to accomplish thirty times what one cell can accomplish, are able to accomplish very much more than this. To this discrepancy between the work that thirty cells can do and the work they might be expected to do, on a purely quantitative basis, the term communal activity has been applied. For the present its nature cannot be more accurately defined.

SUMMARY.

1. The behavior of a single bacterial cell toward gentian violet differs fundamentally from that of a small group of cells (thirty).

2. The explanation of this phenomenon is not purely quantitative; thirty cells accomplish much more than thirty times what one cell can accomplish.

EXPLANATION OF PLATES.

PLATE 78.

Fig. 1. Single unstained cells planted on gentian violet agar; no growth.

Fig. 2. Groups of unstained cells of a gentian-negative strain of *B. coli* planted on gentian violet agar. The smaller groups did not grow. The number of cells in the group planted is indicated on each tube.

PLATE 79.

FIG. 3. Transplantation of single cells on plain agar for control. In Divisions 1 and 2 two separate plants (*a* and *b*, *c*, and *d*) were made, in the other divisions only one. Six growths were obtained out of seven plants.

FIG. 4. Transplantation of single cells of *B. coli* on gentian violet agar. One cell was planted in each of the ten divisions. No growth occurred.

FIG. 5. Transplantation of single cells of *B. coli* on gentian violet agar. A single cell was planted in each division. *A*, barely visible, *B*, invisible at the end of 24 hours. These two colonies are the only two growths which have ever occurred from single cells in gentian violet media.

PLATE 80.

FIG. 6. Single cell transplantations into broth. Strain Z, a gentian-negative strain from a single cell, was used for the seeding. Growth is represented by shading. 80 per cent positives were obtained in the control series (upper row), no positives in the gentian violet broth series (lower row).

FIG. 7. Group transplantations of a gentian-negative strain of *B. coli* (Strain Z) into gentian violet broth. The number of cells seeded is indicated on each tube. Groups below twenty-seven did not grow. Compare the growth of the larger groups with the failure of single cells to grow (see Fig. 6).

THE ANILIN DYES IN THERA-
PEUTICS

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THE ANILIN DYES IN THERAPEUTICS *

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NEW YORK

Of the various features of the anilin dyes which make them an attractive subject for experimental investigation, I will consider the three with which my own studies of gentian violet (and more recently of acid fuchsin) have been chiefly concerned.

THE SELECTIVE POWER OF THE ANILIN DYES

One of the most striking examples of selective bacteriostatic power is furnished by gentian violet. The behavior of bacteria toward this dye follows pretty closely the Gram reaction, and this feature is characteristic also of other triphenylmethane dyes, among them magenta.[1] These facts are now well known; but I have recently been able to show that the behavior of bacteria toward acid fuchsin (a sulphonated triphenylmethane) is, in certain respects, the exact reverse of their behavior toward the basic dyes. If bacteria are exposed to acid fuchsin and planted on plain agar, it is found that it is now the gram-positive spore-bearing aerobes which survive, and the commoner gram-negative bacteria which die. A plate stroked, for example, with *Bacillus paratyphosus* and *Bacillus pseudo-anthracis* which have been exposed to acid fuchsin will show vigorous growth of the latter, and none whatever of the former; while if the experiment is done with gentian violet or magenta there will be vigorous growth of *B. paratyphosus,* and none whatever of *B. pseudo-anthracis* (Fig. 1). I have previously shown that it is quite a simple matter to rid a culture of gram-negatives of contaminating gram-positives by exposing the mixture of the two organisms

to gentian violet, previous to plating; and it is equally easy to rid gram-positive spore bearers of contaminating gram-negatives by exposing the mixture to acid fuchsin. One can thus quite easily from a mixture, let us say, of *Bacillus pyocyaneus* and *Bacillus megatherium,* pick out at will either organism in pure culture by the use of these two dyes (Fig. 2).

It proves, moreover, to be the case (experiments with sulphanilic acid, chromotropic acid and ethylsulphonic acid furnished the proof) that it is the sulphonic acid group in the acid fuchsin which accounts for this reverse selective power. We have succeeded, therefore, for the first time in determining which part of a dye molecule is responsible for its selective activity, and it is interesting that this portion is colorless (Figs. 3, 4 and 5).
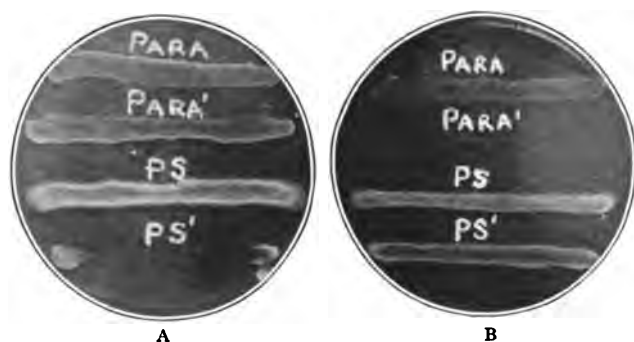
Fig. 1.—Plates stroked with B. parathyphosus and B. pseudo-anthracis which have been exposed to magenta (*A*) and acid fuchsin (*B*): *Para*, B. parathyphosus, unstained for control; *Para'*, stained B. parathyphosus; *P S*, B. pseudo-anthracis, unstained for control; *P S'*, stained B. pseudo-anthracis. In Plate A (magenta), stained parathyphosus has been unaffected; stained pseudo-anthracis has been killed. In Plate B (acid fuchsin), stained parathyphosus has been killed; stained pseudo-anthracis is unaffected.

The facts just referred to would seem to bring the problem of the Gram reaction very much nearer solution. Since opposite selective activities have been demonstrated for two dyes (magenta and acid fuchsin), and since the chemical group in the latter dye responsible for its selective activity has been determined, data would seem to be at hand for a chemical explanation of the difference between the two types of organism.

The facts just cited are significant for the problems of chemotherapy in the following respects:

1. It is clear that the cleavage of bacteria represented by the Gram reaction may have therapeutic importance. This matter is not so simple as appeared to be the case when the parallelism between Gram reaction and gentian violet reaction was first observed. One of the facts which complicated matters was brought to light by the observation last year of two strains within a pure culture of *Bacillus coli*. Both of these were gram-negative. Yet one of them grew well in the presence of gentian violet, while the other would not grow at all. This is one of the pioneer observations of microbic dissociation, as it has since come to be called.[2]
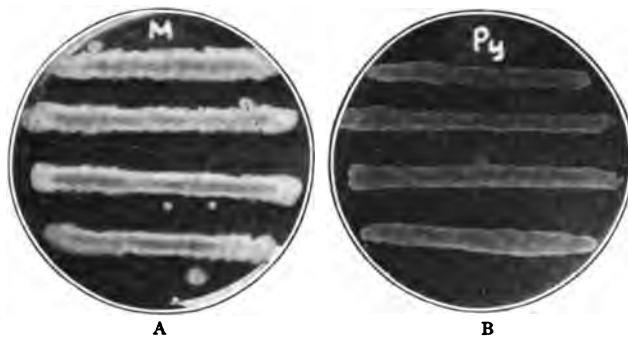
Fig. 2.—Method by which either of two organisms desired may be isolated from a mixture of gram-positive spore bearers and gram-negative bacteria by exposing the mixture to acid fuchsin or gentian violet. Plate A has been stroked with the mixture after exposure to acid fuchsin: a pure culture of B. megatherium results. Plate B has been stroked with the same mixture after exposure to gentian violet; a pure culture of B. pyocyaneus results.

It is clear, too, that the recent observations with acid fuchsin make it necessary to modify somewhat my published statement that "gram-negative organisms are less susceptible to many—possibly to all—selective bacteriostatic substances than gram-positive organisms"; for, as compared at least with the spore-bearing aerobes, they are not less but more susceptible to acid fuchsin. But these facts only emphasize the soundness of the principle of selective bacteriostasis

2. Churchman, J. W.: J. Exper. Med. **33**: 569 (May) 1921.

along the line of cleavage indicated by the Gram reaction.

What is the fundamental nature of these selective activities? An easier penetrability of the gram-positive organisms has frequently been assumed, to explain their susceptibility to the triphenylmethanes. But acid fuchsin, as well as gentian violet, penetrates the gram-positive spore bearers more readily than it does the gram-negatives; yet it does not kill them, though it kills the negatives. Moreover, gentian violet penetrates the spores little if at all, yet they are highly susceptible to it; and gram-negatives may be deeply stained without killing them. Something other than penetration must therefore be concerned. It is tempting to assume that electrical adsorption plays a part,

A   B
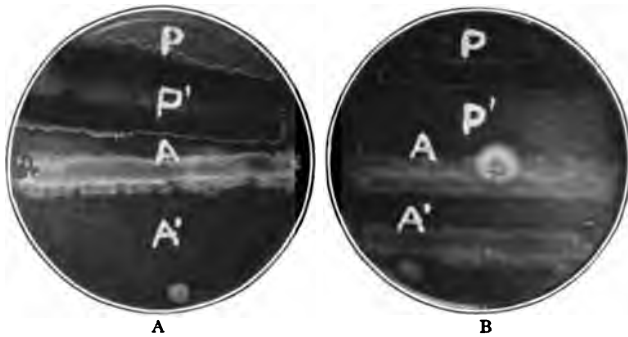
Fig. 3.—Opposite selective powers of sulphanilic acid and gentian violet: Plate A, gentian violet; Plate B, sulphanilic acid; P, B. prodigiosus unstained for control; P', B. prodigiosus stained; A, B. anthracis unstained for control; A', B. anthracis stained.

particularly since basic dyes—which are electropositive—behave in one way toward bacteria, while acid dyes (which are electronegative) behave in certain significant respects in exactly opposite ways. These leads would seem to point the way to a better understanding of the chemistry of the spore and of the mechanism of the Gram reaction.

2. Anilin dyes, however, are not only selective as between species of bacteria; they are also selective as between bacteria and tissue cells. It was shown in 1913 that the living nucleus can be stained by gentian violet, a dye which kills many organisms.[3] Later it

3. Churchman, J. W.: Proc. Soc. Exper. Biol. & Med. 9: 120, 1913-1914.

was shown by injections of the dye into the human knee joint during life that the nuclei of the synovial membrane may be stained without injury.[4] Complete experimental evidence has thus for the first time been brought for the principle that agents which kill bacteria may be innocuous for the vital part of tissue cells, even though they have penetrated it.

I have already indicated by clinical studies some of the possibilities of selective chemotherapy by showing, in a study of amputation wounds at the Walter Reed Hospital, that it was comparatively easy to eliminate the gram-positive *Bacillus diphtheriae* from granulating wounds with gentian violet, but extremely difficult to
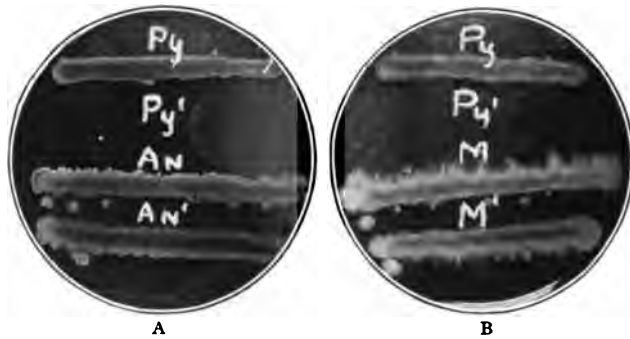
Fig. 4.—Reverse selective power of chromotrophic acid. *Py*, B. pyocyaneus, unstained for control; *Py'*, B. pyocyaneus, exposed to chromotropic acid; *An*, B. anthracis, unstained for control; *An'*, B. anthrasis, exposed to chromotropic acid; *M*, B. megatherium, unstained; *M'*, B. megatherium, exposed to chromotropic acid. The gram-negatives have b.en killed; the gram-positives are unaffected.

get rid of the gram-negative *B. coli*. This was a deliberate attempt to apply clinically the principle of selective bacteriostasis along the lines indicated by laboratory studies, and it was a successful attempt. A further step which might be taken in the same direction is suggested by the recent studies with acid fuchsin. This dye kills *B. pyocyaneus,* an organism which frequently occurs as a troublesome secondary invader of wounds, and which is almost unaffected by gentian violet. There may well be many other similar instances of selective activity; and since the number of bacteria is large and the number of dyes

4. Churchman, J. W.: Treatment of Acute Infections of the Joint by Lavage and Direct Medication, J. A. M. A. **70**: 1047 (April 13) 1918.

and related substances huge, the possibilities seem sufficiently great. The demonstration that gram-negatives are more susceptible to acid fuchsin than gram-positive spore bearers, and the successful isolation of the chemical group responsible for this reverse selective power, open up new lines of approach in this field.

BACTERIOSTASIS

Since the time of Ehrlich, *sterilisans magna*—the destruction of all invading organisms at one stroke—has been the ideal of the therapeutics of infection. If the substances capable of producing such a result were always nontoxic to the host, only that ideal should be pursued. But this is not the case; and the question arises whether there may not also be a place in
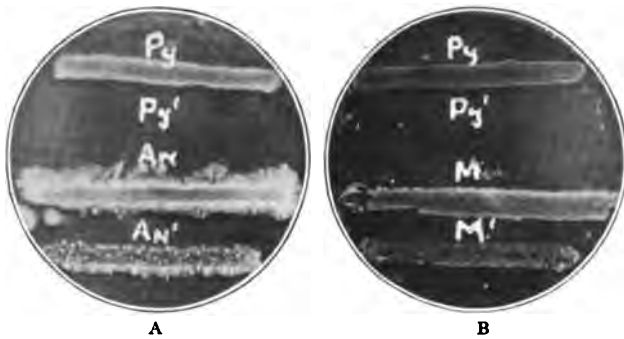
Fig. 5.—Reserve selective power of ethyl-sulphonic acid. Notation same as in Figure 4. The gram-negatives have been killed; the gram-positives are unaffected.

therapeutics for the principle of bacteriostasis: for the use, that is to say, of substances which—in dilutions too weak to do the slightest damage to tissue cells—prevent the growth of bacteria (though they may not kill them) and thus give the tissues a chance to recover of themselves.

Gentian violet possesses both bactericidal and bacteriostatic properties. The former are well brought out by means of divided plates, and its possibilities for technical tricks in bacteriology are numerous. A contaminating colony on a Petri dish, for example, may be readily controlled by ringing it with the dye (Fig. 6), and separation of gram-positives in pure culture may be accomplished with great nicety by growing the

organisms on mediums containing the dye. In the case of gentian violet, bactericidal and bacteriostatic properties run parallel; but this is not true of acid fuchsin and related substances, a puzzling fact for which an explanation has been suggested but not established.

Whatever the mechanism of bacteriostasis may be, it would seem to be a sound therapeutic principle. I have shown that successful use may be made of the principle in the sterilization of acutely infected joints and in ridding stumps of an infection with *B. diphtheriae*. In the joint—a closed cavity—the problem is relatively simple; but one should not expect too much (in spite of some encouraging results already reported) from the use of similar methods in infections of the chest, in which the problem is enormously complicated.

<center>A   B</center>
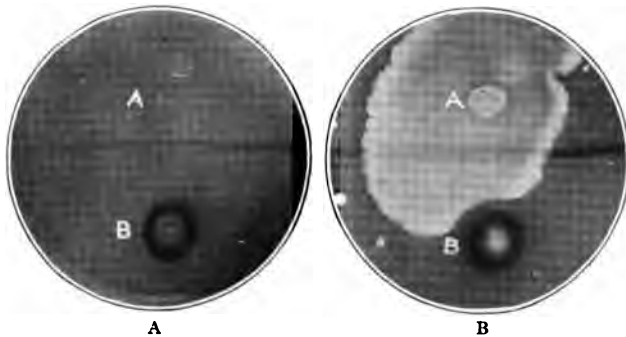
Fig. 6.—Method by which a contaminating colony of B. subtilis may be restrained by ringing it with gentian violet. At *A* and *B* (Plate A) inoculations of B. subtilis have been made; *B* has been ringed with the dye. Plate B shows the same Petri dish after twenty-four hours' incubation.

Yet even in the chest there seems to be no good reason why the well-known benefits to be derived from aspiration alone, in the early stages of infection, cannot be supplemented by mechanical cleansing and the injection of bacteriostatic agents.

PENETRATION

It is the penetrative power of anilin dyes which, more than any other characteristic, has attracted attention to them. Many observers have noticed that, if injected into a cavity such as the bladder, these dyes could be found in the deeper epithelial cells. My own observation is the only one that has been made on the

living human being. Previous to a midthigh amputation I injected the knee joint with gentian violet according to the technic and with the apparatus described for the treatment of acute arthritis. The synovial membrane was prepared for microscopic study by a special technic, and in it the gentian violet could be seen to have penetrated down to the subendothelial layers.

Nor is there any doubt about the ability of dyes to penetrate living bacteria themselves. This has been proved by adding gentian violet to a suspension of *B. coli,* and picking out small groups of the organisms by the Barber technic. These deeply stained individuals grow readily when planted on plain agar, and must therefore have been stained while alive. The significance of the observations already described on the behavior of acid fuchsin and related sulphonic acid substances must be referred to in this connection. When we speak of penetration of bacteria by a dye, we think of permeation of the cell by the whole dye molecule or at least by the visible colored portion of it. It may be, however, that union between the bacterium and the uncolored—though active—portion of the dye has occurred. In the case of acid fuchsin, for instance, the sulphonic acid group is responsible for the dye's reverse selective action. This group is colorless and would not be seen in the organism, though proof of penetration of the gram-negatives is furnished by their death.

LIMITATIONS OF ANILIN DYES

A few of the advantages of the anilin dyes have been pointed out; it remains to consider some of their limitations. They are not very powerful bactericides, though they lend themselves to combinations with substances which are, and so may provide these with their permeating qualities. But this is a procedure which must have sharp limitations. The dyes are chosen for this kind of chemical manipulation because they are not toxic for tissue cells. The substances, like mercury, with which they are usually combined are toxic; and the combinations must therefore be made with great nicety if their purpose is not to be defeated.

Moreover, the activity of the dyes may be impaired by substances in their environment. The inhibitory

effect of serum is well known, and there may be other deterrent influences not now recognized. I have shown, for example, that we are not justified in assuming that substances applied to infected surfaces necessarily reach the invading organisms; the secretions may provide a mechanical barrier against the antiseptic as effective as a layer of petrolatum. If one is working with colorless substances, this fact may not be appreciated; but with dyes, whose course can be followed with the eye, the fallacy of applying antiseptics without preliminary cleansing becomes at once apparent. For treating joints an apparatus has been devised which provides a method of satisfactory preliminary cleansing.[5]

Moreover, the dyes are unstable. Gentian violet injected into the blood stream disappears within two hours; it is incorrect to assume that it circulates very long as such. Perhaps the blood stream could be kept saturated, by repeated injections, with a bacteriostatic substance, and the course of a septicemia thus be checked; but there is no evidence at present that anything like a *sterilisans magna* can be achieved by dyes.

CONCLUSION

The investigation of the problems of infection by a study of the anilin dyes is bound to lead us forward. What we are after is not only the establishment of the therapeutic value of a particular substance (if the principle of selective sterilization becomes established, more than one substance will be used and the substances used may not be dyes at all), but also a clearer understanding of the whole mechanism of selective chemotherapy. And this goal we shall certainly reach.

5. Churchman, J. W.: Sterilization of Closed Cavities by Lavage and Staining with Gentian Violet, J. A. M. A. **77**: 24 (July 2) 1921.

A SEROLOGICAL STUDY OF THE GONOCOCCUS GROUP[1]

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The primary purpose of this investigation has been to determine whether gonococci may be distributed among a number of fixed immunological types or whether, on the other hand, the strains exhibiting dissimilar serological characteristics may more logically be considered as more or less labile variants from a common basal type.

In 1907, one of us (1) published the results of a similar study of this group and arrived at the conclusion that based on agglutination and agglutinin absorption tests, the gonococcus group embraces a heterogeneous collection of types. Among the ten strains studied, three types were recognized to which, however, only six of these strains could be referred. It was realized at the time that the serological relationships within the group were complex and that there existed "all manner of intermediate forms and variations." About this time it was shown by Teague and Torrey (2) that complement fixation tests with these cultures added confirmation to our conclusions in regard to the absence of serological uniformity in this group and indicated the desirability of using several different gonococcus strains in preparing antigens for use in clinical diagnosis.

In view of these findings Dr. H. J. Schwartz and Dr. Archibald McNeil in connection with their investigation of the clinical application of complement fixation tests to the diagnosis of gonococcus infections (3) utilized such of our strains as were then

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available. Of the twelve strains which they received from us, six (A, B, C, G, H, J) had been subjected to serological analysis and six had been subsequently added to the collection without special study. Nine of these strains with one other, S, the origin of which is not known, have been carried in various laboratories to the present time under the designation of "Torrey strains," and have been used quite extensively in the preparation of gonococcic antigen for complement fixation work. These details are published here because of the rather widespread impression that these ten strains had been found to represent ten different gonococcus types. This, however, has never been claimed to be the case as reference to the original articles will show.

That the determination of the serological unity or diversity of strains embraced within a bacterial species of pathogenic propensities is of more than academic interest, is evidenced by the large amount of labor which has been devoted to the study of "types" within the pneumococcus, meningococcus, typhoid, tetanus, influenza and other bacterial groups. The results of some of this work has had a very practical bearing, not only on specific therapy, but also on the epidemiological aspects of these infectious diseases. Up to the present time only a comparatively few serological studies of the gonococcus group have appeared in the literature and to these reference will be made later on.

As regards this present study the particular objectives held in view have been the selection of strains most suitable for complement fixation antigens, the determination of the most representative strains and those with marked antibody stimulating propensities for use in stock vaccines, and also the possible application of these selected strains to the production of curative sera. In addition, of course, our attention was directed to certain epidemiological considerations such as the relationship of strains causing vulvovaginitis in children to those giving rise to gonorrhea in adults.

METHODS

Media. A description will be given in this article only of the media used directly in connection with this serological study, while the cultural procedures found especially efficacious in the isolation of the gonococcus and in the maintenance of stock strains will be dealt with in another communication.

The following ascitic agar medium, which for convenience we have designated as 12 (a), has been used exclusively in obtaining growth for the agglutination experiments. Uniformity in constitution and reaction has been maintained as far as possible. This solid medium yields an unusually heavy growth for gonococcus and generally one which emulsifies readily. Large slants in tubes 1 by 8 inches in size have been used.

Fifteen hundred grams of fresh, fat-free, finely chopped veal are mixed with $2\frac{1}{2}$ liters of distilled water and placed in a covered pot over a low flame. The temperature is gradually raised to the boiling point and the medium allowed to simmer for twenty minutes, with occasional stirring. It is then strained (with pressure) through canton flannel, cooled and the fat removed. It is next placed in a double boiler over a saturated brine bath, the temperature raised to about 60°C. and 1 per cent peptone (Difco), 2 per cent fresh urine, 2 per cent glycerine, 0.5 per cent NaCl and 2 per cent flaked agar are added. This mixture is allowed to boil for forty-five minutes. The reaction is then adjusted to pH 6.9 (using a 10 per cent solution of sodium carbonate), and the boiling is continued for thirty minutes. The loss from evaporation up to $2\frac{1}{2}$ liters is replaced with distilled water. It is next filtered through canton flannel and about 15 cc. are placed in each of the large tubes noted above. Sterilization is effected by heating in the autoclave at 12 pounds pressure for 10 minutes. In preparing the slants ascitic fluid, free from bile, is added in the proportion of one part to four or five of the above medium. The final reaction is generally close to pH 7.2. The slants should not be prepared longer than the day before they are to be used.

The collection of gonococcus strains has been maintained on a

semi-solid vitamine medium prepared, with a slight modification, according to the formula given by Huntoon (4). The marked advantages of this medium in carrying a large series of gonococcus cultures will be discussed in another place.

The medium used in connection with the preparation of antigen for the complement fixation experiments is described in the section dealing with that work.

Animal immunizations. One of the first questions considered in connection with this study was the type of gonococcus antigen which possessed the best agglutinogenic properties. Comparative tests were first made with living and (heat) killed gonococci. The results of a test of this character are given in table 1. The two rabbits received exactly the same dosage of antigen, given at the same time intervals; the only difference lay in the fact that to one (222) living antigen and to the other (223) antigen heated at 55°C. for fifteen minutes were given.

Perry and Kolmer (5), in a comparative study of the immunizing properties of *B. typhosus* vaccine treated in various ways, reported that, as far as their agglutinogenic potentialities were concerned, they might be ranked in the following order: living and autolyzed; mercophen and tricresol killed; heat killed; and lastly, alcohol killed sensitized sediment. We have conducted some similar experiments using gonococcus strain 33 and killing the antigen through the use of (a) tricresol, 0.25 per cent, (b) acetone, (c) chloroform, (d) heated at 55°C., and (e) at 65°C. for fifteen minutes. Rabbits were immunized with equal amounts of each of these variously treated antigens and, after six inoculations, agglutination tests were carried out with the homologous strain. Much better results were obtained with the heat-killed and also with the chloroform treated antigens than with those treated in the other ways specified. It was decided, then, as a routine procedure to use antigens exposed to 45°–50°C. for fifteen minutes, as this degree of heat was found sufficient to kill the gonococci and would be likely to cause no change in its antigenic properties.

Some of the rabbits used in the production of the immune sera were injected at weekly intervals and others were given more intensive treatment by inoculations on three consecutive days a

intervals of four to seven days. The latter procedure yielded much the better results, both as regards the titer of the serum produced and the time consumed. All of the injections were given intravenously. The amount of the dosage of standard

TABLE 1

Comparative agglutination test with serums of rabbits immunized with living and heat (55°C.) killed gonococcus antigen. Rabbit 222 received living strain 41. Rabbit 223 received killed strain 41. The experiment was conducted at 55°C. and the results read after twenty-four hours.

STRAINS	SERUM NUMBERS	NORMAL SERUMS	AFTER 3 INOCULATIONS	AFTER 9 INOCULATIONS
1	222	<25	50	100
	223	<25	100	100
5	222	<25	50	250
	223	<25	100	250
9	222	50	1500	3000
	223	50	1500	3000
18	222	<25	100	1500
	223	<25	250	1000
20	222	<25	100	2000
	223	<25	500	1500
25	222	<25	25	100
	223	<25	25	100
41 Homologous	222	<25	250	2000
	223	<25	750	1500

This experiment indicated that there was little choice between living and heat-killed agglutinogen, although after nine inoculations a slightly higher titer serum was obtained with the living culture.

suspensions was regulated entirely by the condition of the animal. The sera were preserved, for the most part, with 0.1 per cent phenol.

Preparation of antigens for agglutinations. In preparing the bacterial suspensions for agglutination experiments, a standard procedure was carefully followed. The gonococcus strains were grown on the ascitic agar (12a) which has been described. It

was found advantageous to seed these slants from twenty-four hour growths on the same medium. They were incubated at 36° to 37°C. for twenty-four to forty-eight hours. Before emulsifying the growth, the water of condensation and hysteresis was removed by washing out with normal saline solution. This saline solution was prepared with chemically pure NaCl, 0.85 per cent in distilled water. In some of the experiments 0.5 per cent formalin was added to the salt solution, both because this agent preserved the gonococci perfectly for an indefinite period and also in that it tended to check spontaneous clumping. Although Sands (6) found in connection with typhoid agglutination experiments that especially satisfactory results were obtained if the bacilli were emulsified in saline containing 1 to 2 per cent formalin and Hooker (7) also advocates formalinized saline for typhoid agglutination, it has been our experience with the gonococcus that even 0.5 per cent of formalin causes a considerable decrease in sensitiveness to the action of agglutinin, which is much more marked in connection with some strains than in that of others. Such being the case formalinized emulsions were discarded for freshly prepared living suspensions. Gordon (8) and a number of other investigators of serological relationships within the meningococcus group have used emulsions heated at 65°C. for one-half hour, to destroy the autolysin, with 0.5 per cent phenol added as a preservative. It has been our experience that this degree of heat is insufficient to affect greatly the gonococcus autolysin. McClintock and Clark (8) have also reported that temperatures of less than 70°C., with or without the addition of tricresol up to 0.4 per cent, do not prevent the disintegration of suspensions of gonococci in salt solution. With certain recently isolated strains we have found that heating for one-half hour at 65°C. increases markedly the viscocity of the emulsion. Sands (6) noted that heating the typhoid bacillus at 60°C. generally increased their susceptibility to specific agglutinins. We have not noted, however, any enhanced sensitiveness to agglutinins on the part of gonococci after treatment with heat. As there is a good deal of risk of affecting the antigenic properties by heating at 70°C. or higher, and other methods for

destroying the autolysin seemed still more objectionable, we have found it necessary to use living untreated emulsions.

Standardization of gonococcal suspensions. In order that agglutination tests within bacterial groups may have definite comparative value, it is necessary that the density of the culture suspensions be carefully standardized. To obtain such uniformity in the dosage of antigen we adopted the following procedure: After washing and emulsifying the twenty-four to forty-eight-hour growth in the saline solution, as described in the preceding section, the suspension of gonococci was filtered through washed absorbent cotten into a test tube 18 mm. in diameter. Saline was then added until the density of suspension was such that the edge of a wax pencil mark on the back of the tube was just definitely discernible by the observer with his back to a good light. This suspension gave a count of approximately three billion organisms per cubic centimeter, which, when mixed with equal parts of the serum dilution, was reduced to about 1½ billions. In setting up the agglutination tests a rather narrow (8 mm. inside diameter) serological tube was used, and to 0.5 cc. of the serum dilution, 0.5 cc. of the bacterial suspension was added.

Incubation and reading of the tests.—Incubation of the tests was conducted at 50° to 55°C. through the use of a water bath. This temperature is preferable to 37°C. in that it accelerates the reaction and also, in some degree, tends to inhibit spontaneous clumping. During the early part of the experimental work the tubes were kept at this temperature for eighteen to twenty-four hours and then read, but later, after an incubation of two hours, the tubes were placed over night in the ice-chest; there was no appreciable difference in the results. Readings were made at the one-, two- and eighteen- to twenty-four-hour periods. The last period reading was adopted as the final result but the earlier observations often yielded important information, especially when spontaneous precipitation was a confusing factor.

This tendency to spontaneous clumping on the part of certain strains of gonococci was a troublesome feature in the agglutination work and no way was found to eliminate it entirely with

certain strains. This tendency was not confined to certain old stock strains but was noted, in a few instances, as a characteristic of recently isolated ones. The saline control tube gave, of course, a clue to the amount of non-specific clumping and precipitation, but the best method for discounting this factor lay in a careful inspection of the character of the sediment in tubes showing apparent agglutination. If the sedimentation was non-specific, a gentle shaking of the tube broke up the clumps and brought about an even suspension of the cocci. With true agglutination, on the other hand, the clumps are generally large, firm and compact, and are not disintegrated by a fairly sharp shake of the tube; as a rule, the more complete the agglutination, the larger and more firmly united are the clumps. It should be added that spontaneous agglutination was an annoying factor in connection with only a small minority of the strains. At times it was found advantageous, after making a reading at the twenty-four hour period, to shake up the tubes and reincubate them for a few hours. Following this procedure the end point was sometimes more sharply indicated.

The end point selected, as marking the agglutination titer, was the last dilution tube showing complete or almost complete clumping and sedimentation of the organisms; a tube given this reading showed either a clear condition or no more than a very faint clouding of the supernatant fluid (the latter reaction was read as + + +). Reactions of less degree than this are deceptive in character and do not serve as a trustworthy guide to the true titer of a serum.

SOURCE OF THE GONOCOCCUS STRAINS

This collection of gonococcus strains is quite unique in that the infection of a considerable number of cases, from which successful isolations were made, was known to have occurred in widely separated foreign countries and also in different parts of this country. Our strains, perhaps, are thus more nearly representative of the gonococcus group than any collection which has been studied serologically heretofore. The origin of these cultures, where it is of interest, will be noted in subsequent sections.

The ten so-called "Torrey strains" are designated by the numbers 24, 29, 30 to 37. These strains have been under cultivation for about fourteen years; other strains of about the same age are numbers 21 and 23.

As regards the type of infection from which these strains were isolated, forty-seven were from cases of acute and chronic urethritis of males, four from genito-urinary infections of females, four from joint cases, two from septicaemias, one from an eye infection and thirteen from vulvovaginitis infections of children.[2]

AGGLUTINATION

With a number of bacterial species agglutination tests alone, without absorptions, have been found to serve as a quite trustworthy guide to their distribution among serologically distinct types. Such tests, for instance, are generally sufficient for the typing of pneumococci and also, according to some investigators, even the meningococci. In reference to the latter, Butterfield and Neill (9) have reported that 90 per cent of their meningococci strains could be typed by straight agglutinations alone, using Gordon's type serums prepared with Gordon's four type cultures. With the gonococcus group, however, straight agglutination tests have yielded almost no clue to specific serological relationships.

In table 2 are reported some agglutination results with our first forty-seven strains. The figures in the first five columns are particularly comparable in that the rabbits immunized respectively against strains 33, 1, 5, 8 and 13 had received ex-

TABLE 2

Agglutination reactions of gonococcus cultures with gonococcus sera. Rabbits 218, 219, 220, 221 and 861 had received identical treatment as regards the number (12) and dates of inoculations and the dosage. The homologous titers are in heavier type*

STRAINS	RABBIT SERA IMMUNE TO GONOCOCCUS STRAINS							
	33 (rabbit 218)	1 (rabbit 219)	5 (rabbit 220)	8 (rabbit 221)	13 (rabbit 861)	29 (rabbit 217)	41 (rabbit 223)	7 (rabbit 230)
1	<25	100	50	0	0	100	<50	<100
2	0	100	<25	0	0	0	<50	<100
3	0	2000	50	100	250	250	250	4000
4	50	<25	<25	25	250	·0	0	1000
5	50	100	250	25	50	<50	100	100
6	<25	1000	50	100	750	250	500	1000
7	50	250	100	0	25	500	250	3000
8	25	250	250	250	250	<25	250	1000
9	3000	3000	250	1000	1000	1500	2000	
10	1000	250	100	250	50	500	500	2000
11	2000	700	750	750	2000	250	1500	1000
12	100	50	50	25	250	<50	50	100
13	<25	50	25	0	25	<50	<50	<100
14	750	100	25	100	100	25	1000	100
15	1000	1000	<25	750	250	500	500	2000
16	100	250	25	<25	100	0	100	<100
17	250	100	250	25	100	25	100	<100
18	100	100	500	750	100	25	50	100
19	2000	700	100	250	2000	1500	1500	<100
20	750	50	<25	50	100	100	100	4000
21	250	<25	750	50	100	500	100	<100
22	0	50	25	0	50	<25	<50	<100
23	1000	250	750	750	5000	1500	1500	4000
24	0	50	25	250	0	1500	250	2000
25	100	250	250	500	100	0	<50	<100
26	25	1000	25	25	0	50	<50	100
27	<25	50	50	250	25	0	50	<100
28	0	0	<25	<25	0	0	<50	<100
29	750	250	25	<25	1000	500	1500	3000
30	750	1000	1000	250	750	500	2000	4000
31	750	25	250	<25	250	1000	1500	4000
32	2000	3000	1000	500	750	3000	2000	4000
33	1000	1000	750	250	750	1500	1000	4000
34	1000	700	1000	250	250	1000	3000	4000
35	250	700	2500	250	100	1500	1000	4000

* A small dosage was used in the immunization of all these five rabbits.

TABLE 2—*Continued*

STRAINS	RABBIT SERA IMMUNE TO GONOCOCCUS STRAINS							
	33 (rabbit 218)	1 (rabbit 219)	5 (rabbit 220)	8 (rabbit 221)	13 (rabbit 861)	29 (rabbit 217)	41 (rabbit 223)	7 (rabbit 230)
36	<25	1000	250	250	750	1000	2000	3000
37	1000	1000	1000	1000	2000	<50	500	2000
38	500	250	100	500	750	50	250	1500
39	50	<25	250	50	25	<50	250	<100
40	250	700	100	100	750	0	50	1500
41	25	500	50	100	0	<50	250	500
42	750	500	750	750	750	<50	1500	4000
43	50	100	<25	100	<25	0	50	<100
44	<25	100	250	50	0	100	50	1000
45	0	250	25	500	500	<50	100	1500
46	<25	100	25	100	<25	0	50	<100
47	1000	250	50	100	250	<50	500	3000

actly similar treatment as regards dosage, inoculation intervals and time of bleeding.

The inconsistencies, as far as possible typing is concerned, revealed by straight agglutination tests, may be illustrated by the reactions of strain 33 and its serum (218). This serum agglutinated strains 1, 5, 8 and 13 only at a dilution of 1:25 or less and yet a serum produced to each one of these strains agglutinated strain 33 in dilutions as high or much higher than their respective homologous titers (table 2). An inspection of this table also shows that, with the exception of strain 8 with strain 7 serum, these strains agglutinated poorly with all of the eight sera and their failure to react with strain 33 serum may be as reasonably ascribed to their poor agglutinating qualities as to the absence of specific or group agglutinins to which they are sensitive. Straight agglutination tests, then, are not of any definite value in indicating possible type relationships between gonococcus strains. Warren (11) in a recent study of the agglutination reactions of twenty-three gonococcus strains came to a similar conclusion, but for the reason that all the strains agglutinated about equally well with the three or four sera tested.

Gonococci are reputed, as a class, to be more sensitive to the action of agglutinins than are the meningococci. Although this

may be in a measure true in that a much higher titer may be
obtained through inoculations with certain gonococcus strains,
especially those cultivated for some time, than is possible with
meningococci and their respective anti-sera, yet poorly agglutin-
ating gonococcus strains have been quite frequently encountered,
especially when tested soon after isolation.   Among sixty-three
recently isolated strains, twenty-six were found to be relatively
inagglutinable with the test sera.   Of course failure to react
may have been due in some instances to absence of group agglu-
tinins to which the strains were sensitive, but with this reserva-
tion the fact that 42 per cent of these gonococcus strains were
apparently relatively inagglutinable corresponds closely with
the 40 per cent of similarly inagglutinable meningococcic strains
reported by Elser and Huntoon (10) as occurring in their series
of cultures.   Some gonococcus strains, relatively inagglutinable
soon after isolation with the sera employed, became later much
more sensitive to the action of agglutinins, whereas other strains,
such for example as 2, 5, 13, 16, 22, 28 and 46, did not acquire
under cultivation any increased susceptibility to the action of
agglutinins.   We have, however, encountered no instance of a
strain becoming inagglutinable under cultivation as was reported
by Elser and Huntoon to have occurred in reference to certain
of their meningococcic strains.   Attempts were made to increase
the sensitivity of some of our inagglutinable strains, but neither
growing them in glucose broth for several generations—a method
applied successfuly with meningococci by Elser and Huntoon—
nor by a period of daily transplants, was their agglutinability
enhanced.

Elser and Huntoon (10) observed that, although the most
agglutinable of their strains of meningococci were likely to pro-
duce the more potent agglutinating sera, there was no definite
relationship between agglutinability and agglutinogenic proper-
ties.   We have found this to be true also for gonococci and, as
is shown in table 2, sera produced with certain relatively inagglu-
tinable strains (1, 5, and 13), although giving a low titer for them-
selves, may agglutinate other strains in high dilutions.   These
strains may also be capable of absorbing well.

It has been the common experience of those who have studied extensively the agglutination of the meningococcus and also of the gonococcus that the agglutinability of a given strain may exhibit a considerable degree of variation when tested from time to time with the same serum.   These variations are probably

TABLE 3

Agglutination of recently isolated gonococcus strains and relatively inagglutinable stock strains with a serum immune to strains 15 and 41.   Titer for homologous strain 15 = 30,000.   Titer for homologous strain 41 = 2000.

RECENTLY ISOLATED STRAINS							RELATIVELY INAGGLUTINABLE STOCK STRAINS					
Strains	Generation number	Serum dilutions					Strains	Serum dilutions				
		100	200	500	1000	2000		100	200	500	1000	2000
J	3	4	4*	3	—	—	1	4	4	2	—	—
01	4	4	4	4	2	—	2	4	4	3	—	—
02	4	4	3	2	—	—	5	4	3	—	—	—
03	4	4	2	1	—	—	12	2	2	—	—	—
04	3	4	4	4	1	—	16	4	4	—	—	—
05	3	4	4	4	4	—	20	4	4	4	4	3
06	4	4	4	3	2	—	21	4	4	4	3	2
07	4	4	4	4	4	2	22	4	2	—	—	—
91334	4	4	4	—	—	—	25	4	2	—	—	—
08	4	4	3	—	—	—	39	4	4	4	3	2
09	4	4	4	3	2	—	45	4	4	3	3	—
010	5	4	2	—	—	—	53	4	3	—	—	—
011	5	4	4	4	4	2	55	4	2	—	—	—
012	4	4	4	4	4	4	56	4	—	—	—	—
013	3	4	2	—	—	—	57	3	—	—	—	—
014	3	3	1	—	—	—	59	4	3	—	—	—
015	3	4	3	1	—	—	62	4	3	—	—	—
016	3	4	4	4	4	3	77	4	4	—	—	—

* 4 indicates complete agglutination; 3, nearly complete agglutination.

referable to slight differences in the physical characteristics of the growth due to slight changes in the condition of the medium or the environment, especially as regards moisture.   In experiments of a comparative nature this tendency of the gonococcus to vary in agglutinability must be constantly borne in mind and controlled with great care.

With a single exception none of the monovalent agglutinating sera gave promise of being of much value for the identification of the gonococcus.   The serum giving the best results was one of a high titer immune to strain 15 (table 5) which agglutinated sixty-seven of the seventy-four strains (or about 90 per cent) in a dilution of 1:250 or higher.   Several polyvalent sera prepared through immunization with two or more selected strains have been fairly effective, but none covered our entire collection of strains.   Perhaps the best combination of any two of our strains is that of 15 and 41.   The results with a high titer serum produced with this combination is shown in table 3.   In this table are given the titers obtained with a series of very recently isolated strains and also that of some of the relatively inagglutinable strains which had been under cultivation for many months. It may be said in general that a positive agglutination with a gonococcic serum at a dilution of at least 1:250 definitely identifies the culture under consideration as gonococcus but that a negative result has no certain significance as some undoubted strains of gonococci are practically inagglutinable.

<center>ABSORPTIONS</center>

The greater part of the time occupied by this study has been devoted to agglutinin absorption tests to determine whether our series of gonococcus strains may be distributed through this method of classification among a number of clear-cut, immunological types or, if not, in what way the various strains are related. In conducting these tests our effort has been to maintain the conditions as nearly uniform as possible.[3]

<center>*Methods*</center>

In order to obtain sufficient growth for these absorptions, the strains were seeded on large potato tube slants of ascitic agar, prepared as heretofore described and with a reaction close to pH 7.2.   Plates were used at first but proved inferior to the

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[3] In much of this absorption work and also in the complement fixation tests we have received valuable assistance from Luther B. Conklin.

slants. These slants had each a surface area of approximately 2500 sq. mm. It was found advantageous to make plantings from twenty-four-hour growths on the same medium. Generally three tubes were seeded with a single strain for an absorption. After twenty-four to forty-eight hours incubation the growths were washed with the saline solution and then suspended in a small amount of the same. The suspension of gonococci was then placed in a graduated centrifuge tube and whirled until 0.2 cc. of rather loosely packed organisms had been deposited in the tip of the tube. In thus measuring the absorbing dose of culture we are following the method used by Valentine and Cooper (12) in their serological study of the *B. influenzae*. This method, as applied to gonococci, is not strictly accurate for comparative experiments owing to a number of uncontrollable factors, such as the amount of autolysis, but is perhaps as satisfactory as any which may be utilized. Generally not all of the growth from the three slants was required for the absorbing dose and the excess was used in determining the agglutination titer of the unabsorbed serum for the particular strain. With every serum an absorption was carried out with this dosage of a meningococcus strain in order to control the factor of non-specific absorption. This test of specificity was of much value as evidence of non-specific absorption occasionally appeared in connection with low-titer sera. The serum to be absorbed was diluted to the proper degree (this should be approximately the lowest dilution at which the homologous strain in the dosage used will completely absorb its agglutinins) and the centrifuge tube containing the sedimented bacteria was filled to the 5-cc. mark with it. An even suspension was then made of the growth in the diluted serum, and the tube placed in the water bath at 45° to 50°C. for two hours, it being shaken at intervals of about fifteen minutes. Unabsorbed serum, diluted to the absorption titer, was exposed to this temperature for the same time period. Both the absorbed and the unabsorbed serum lots were then centrifuged until the former tubes were clarified fully or to a sufficient degree for the purposes of the test. Centrifugation of the unabsorbed serum was considered advisable in view of the finding of Elser and Huntoon

that the vibration of the machine might cause a lowering of its titer. Suitable graded dilutions of the unabsorbed and absorbed lots of the serum were then prepared and agglutination tests set up by which the following titers were determined: that of the absorbing strains with the unabsorbed serum; the absorbing strains with their respective lots of absorbed serum; the homologous strain with each lot of absorbed serum and also with the unabsorbed serum. The last two determinations with the homologous strain are, of course, the all important ones in tracing specific relationships, but the others give much additional information or serve as important controls. The absorption tests were incubated and readings made as described for the straight agglutination experiments.

In a number of recent studies of type determinations among bacterial groups through absorption methods, an absorbing period of three to six hours at 50° to 55°C. has been employed. Hermanies (13), in a study of this character of the gonococcus, incubated his absorptions at 55°C. for four to six hours, left the tubes overnight in the ice-chest, and carried out the tests the next day. As our absorptions and tests were always carried through on the same day, we have employed a two hour absorption period, and believe that the agglutinin is fully bound within that period. This opinion seems substantiated by the comparative test reported in table 4. Some differences in the degree of absorption may be noted, especially marked as regards strain 25, but these discrepancies should probably be referred to the apparently inevitable variations associated with agglutination of gonococci rather than specifically to the absorption time factor. It should be noted that these two tests were not carried out on the same day, but this rather adds to than detracts from their comparative interest.

In the attempts to distribute our gonococcus cultures among distinct serological types, the first fifty strains were used for the most part, although with three sera the tests were extended to include all but a few of the total seventy-seven strains. In all these experiments the absorption of specific or homologous agglutinins was alone determined and the question of relationships

through so-called minor or group agglutinins was not approached. By way of illustration a complete report is made in table 5 of the results with one of the test sera on all the strains then available. It should be noted that the percentages of homologous agglutinin absorptions are stated in several instances in approximate rather

TABLE 4

Table to show the comparative effect of absorptions at the following time periods: A. Absorbed at 55°C. for two hours. B. Absorbed at 55°C. for six hours and left in ice box over-night. Serum 104, immune to culture 15.* Absorbed at dilution of 1:250

CULTURES	TITER OF ABSORBING CULTURE WITH UN-ABSORBED SERUM	TITER OF ABSORBING CULTURE WITH AB-SORBED SERA	TITER OF HOMOLO-GOUS CULTURE WITH ABSORBED SERUM	TITER OF ABSORBING CULTURE WITH UN-ABSORBED SERA	TITER OF HOMOLO-GOUS CULTURE WITH ABSORBED SERA	PERCENTAGE OF ABSORPTION	
	A			B		A	B
1	500	<500	1000	<500	500	87.5	93.8
7	8000	<500	4000	<500	4000	50.0	50.0
11	16000	<500	1000	<500	<500	87.5	100.0
15 Homologous	8000	<500	500	<500	<500	100.0	100.0
18	4000	<500	2000	<500	2000	75.0	75.0
19	8000	<500	500	<500	500	93.8	93.8
25	500	<500	500	<500	4000	93.8	50.0
30	8000	<500	500	<500	1000	100.0	87.5
34	8000	<500	2000	<500	2000	75.0	75.0
41	500	<500	8000	<500	8000	0.0	0.0
49	2000	<500	8000	<500	8000	0.0	0.0
Meningococcus	<250	<500	8000	<500	8000	0.0	0.0

* Titer homologous strain 15 with serum 104 = 8000.

than exact figures; repeated tests would show too many variations to justify the presentation of very exact figures in reference to a single experiment. These percentages, however, give a sufficiently accurate idea of the serological relationships of the several strains to the homologous culture.

As has already been stated this serum 104 immune to strain 15 gave positive agglutinations with a greater number of our strains than any other tested as extensively. An examination of

TABLE 5

Strain 15 serum (104) absorbed with each of gonococcus strains 1 to 77 and with meningococcus, and tested as shown below. Serum absorbed at 1:250 dilution

STRAIN	TITER UNABSORBED SERUM	TITER ABSORBING STRAIN WITH ABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH UNABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH ABSORBED SERUM	PERCENTAGE ABSORPTION OF SPECIFIC AGGLUTININS
1	500	<500	8000	1000	87
2	1000	<500	8000	8000	0
3	4000	<500	8000	2000	75
4	1000	<500	8000	4000	50
5	<250	<500	8000	4000	50
6	8000	<500	8000	2000	75
7	500	<500	8000	4000	50
8	1000	<500	8000	1000	87
10	8000	<500	8000	2000	75
11	4000	<500	8000	<500	100
12	500	<500	8000	1000	87
13	1000	<500	8000	8000	0
14	1000	<500	8000	2000	75
15*	8000	<500	8000	<500	100
16	500	<500	8000	8000	0
17	1000	<500	8000	8000	0
18	4000	<500	8000	2000	75
19	1000	<500	8000	500	93
20	500	<500	6000	6000	0
21	500	<500	6000	4000	33
22	<250	<500	6000	6000	0
23	8000	<500	6000	1000	84
24	8000	<500	6000	<500	100
25	500	<500	8000	500	93
26	1000	<500	6000	1000	84
27	1000	<500	6000	4000	33
28	2000	<500	6000	2000	66
29	8000	<500	6000	500	92
30	8000	<500	8000	<500	100
31	8000	<500	6000	1000	84
32	8000	<500	6000	1000	84
33	6000	<500	6000	1000	84
34	8000	<500	8000	2000	75
35	8000	<500	8000	500	100
36	250	<500	6000	1000	84
37	6000	<500	6000	1000	84
38	4000	<500	8000	4000	50
39	500	<500	6000	2000	66

* Homologous.

TABLE 5—*Continued*

STRAIN	TITER UNABSORBED SERUM	TITER ABSORBING STRAIN WITH ABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH UNABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH ABSORBED SERUM	PERCENTAGE ABSORPTION OF SPECIFIC AGGLUTININS
40	2000	<500	6000	2000	66
41	500	<500	8000	8000	0
42	4000	<500	6000	500	92
43	<250	<500	6000	<500	100
44	2000	<500	6000	500	92
45	250	<500	6000	2000	66
46	<250	<500	6000	2000	66
47	2000	<500	6000	4000	33
49	2000	<500	8000	8000	0
50	4000	<500	6000	1000	84
51	2000	<500	6000	500	92
52	250	<500	6000	500	100
53	250	<500	6000	1000	84
54	2000	<500	6000	2000	66
55	500	<500	6000	4000	33
56	<250	<500	6000	4000	33
57	250	<500	6000	2000	66
58	500	<500	6000	6000	0
59	250	<500	6000	2000	66
60	4000	<500	6000	1000	84
61	8000	<500	8000	500	92
62	500	<500	6000	4000	33
63	8000	<500	6000	500	92
64	8000	<500	8000	2000	75
65	1000	<500	8000	4000	50
66	16000	<500	8000	1000	87
67	2000	<500	8000	8000	0
69	6000	<500	8000	2000	75
70	4000	<500	6000	500	92
71	8000	<500	8000	500	93
72	<250	<500	6000	6000	0
73	2000	<500	8000	500	93
74	8000	<500	8000	6000	25
75	8000	<500	8000	8000	0
76	2000	<500	8000	8000	0
77	250	<500	8000	6000	0
Meningo-coccus	<250	<500	8000	8000	0

table 5, however, shows that a high agglutinating titer with the serum does not necessarily mean a specific relationship to the serum strain; for example strains 43, 52 and 53 gave a titer of

only 1:250 or less and yet these cultures were able to absorb the homologous agglutinins strongly, on the other hand strains 74 and 75 agglutinated to the full titer of the serum and yet absorbed little or none of the homologous agglutinins. Many other examples of a similar discrepancy between agglutinating and absorbing capacities may be found in this table and, in fact, were encountered frequently in the course of this investigation. These facts indicate that straight agglutination tests with gonococci are not as trustworthy a guide to specific relationship as they appear to be for the meningococcus and certain other bacterial groups.

Absorption tests of the same type as that illustrated by table 5 were carried out with nine other sera. The serum strains used were fairly representative of our whole collection and were derived from widely separated geographical localities. It is neither feasible nor desirable to present here a complete tabulation such as has been given for strain 15 serum (table 5). It may be stated, however, that absorptions with each of these sera were conducted and controlled in the same way as for strain 15 serum. It would seem sufficient for the purpose of tracing specific inter-relationships to give only the approximate homologous agglutinin absorption percentages of each of the fifty strains for each of the sera. These results are presented in such a way in table 6 that the specific affinities of the strains may be readily evaluated. It may be stated again that our definite end point, as described on page 312, served as the agglutination titer indicator, that the homologous titer of the serum strain was always determined in connection with each absorption, and that a control absorption with meningococcus was also run with each serum.

In an analysis of table 6 let us first compare the results with the sera 104, 217 and 218, immune respectively to strains 15, 29 and 33. These strains were considered very closely related as the homologous agglutinins produced by each were absorbed by each. The table shows that the specific or homologous agglutinins of these three sera are absorbed in a percentage amounting to 75 or over by twenty-two of the fifty strains. These twenty-two strains (3, 4, 6, 8, 9, 10, 11, 15, 19, 23, 29, 30, 31, 32, 33, 34, 35, 36, 37,

42, 43, 50) may be placed together in one group as far as the results with these sera are concerned. The agglutinin content of these three sera is also similar in that five strains (2, 16, 27, 41 and 49) are incapable of absorbing any of their respective specific agglutinins and closely associated with these are five other strains (5, 25, 28, 39 and 46) which show a specific relationship, and that only partially, to only one of these sera, namely, strain 15 serum. It should be mentioned that the gonococcus strains had been under cultivation for a much longer period at the time of strain 15 serum absorptions than when the tests with the other two sera were made. This, as will be explained later, is an important consideration due to the labile nature of the antigenic constituents of some strains. We have also a residuum of eighteen strains (1, 7, 12, 13, 14, 17, 18, 20, 21, 22, 24, 26, 38, 40, 47, 48) which show partial relationships to the above three serum strains in that they absorbed the specific agglutinins completely or incompletely from one or more of these sera.

Strain 8 showed marked specific relationships to strains 15, 29 and 33, and it would seem reasonable to expect that absorptions of a serum produced with this strain would indicate a similar grouping of the gonococcus strains. This, in large measure, proved to be the case, although there were rather fewer strains in the group absorbing little or none of the homologous agglutinins. With respect to a few strains, also, there are certain marked differences, as for example the fact that strains 27, 28 and 39, which absorbed little or none of the homologous agglutinins from strains 15, 29 and 33 sera, removed practically all of these agglutinins from strain 8 serum; on the other hand, strains 7, 12, and 44 did not absorb the homologous agglutinins from strain 8 serum but did so from the other three sera.

The next serum (106) reported in this table was immune to a strain (42) which showed very close relationships to the four preceding serum strains (29, 33, 15, 8). This strain produced a serum from which the homologous agglutinins were absorbed by a larger number of the test strains than was the case with any other serum investigated. There are thirty-three strains in the 75 to 100 per cent class and only eight in the 0 to 33 per cent

TABLE 6

Percentage absorptions of homologous agglutinins from nine gonococcic sera by 50 gonococcus strains and a meningococcus strain. Geographical source of serum strains: 29, U. S. A.; 33, U. S. A.; 15, foreign (?); 8, Mexico; 42, U. S. A.; 7, Germany; 18, England; 41, Belgium; 5, England

ABSORPTION PERCENTAGES	STRAIN 29 (217) SERUM HOMOLOGOUS TITER: 1000-2500 ABSORBED AT 1:50	STRAIN 33 (218) SERUM HOMOLOGOUS TITER: 1000-2500 ABSORBED AT 1:25	STRAIN 15 (104) SERUM HOMOLOGOUS TITER: 6000-8000 ABSORBED AT 1:250	STRAIN 8 (225) SERUM HOMOLOGOUS TITER: 500-1000 ABSORBED AT 1:25	STRAIN 42 (106) SERUM HOMOLOGOUS TITER: 1500-2000 ABSORBED AT 1:25 and 1:50	STRAIN 7 (230) SERUM HOMOLOGOUS TITER: 2000-3000 ABSORBED AT 1:50	STRAIN 18 (122 and 237) SERA HOMOLOGOUS TITER: 400-600 ABSORBED AT 1:25	STRAIN 41 (223) SERUM HOMOLOGOUS TITER: 500-1000 ABSORBED AT 1:50	STRAIN 5 (95) SERUM HOMOLOGOUS TITER: 500 ABSORBED AT 1:25
75 to 100	3, 4, 6, 8, 9 11, 17, 19 23, 26, 29* 30, 31, 32, 33, 34, 35, 36 42 50	3, 4, 6, 7, 8, 9 10, 11, 12, 13, 15, 19 20, 21, 22, 23, 24, 29 30, 31, 32, 33, 34, 35, 36, 37 42, 43, 47 50	1, 3, 6, 8 10, 11, 12, 14, 15, 18, 19 23, 24, 26, 29 30, 31, 32, 33, 34, 35, 36, 37 42, 43, 44 50	6, 8 10, 14, 15, 18, 19 20, 21, 24, 27, 28, 29 30, 32, 33, 37, 39 42, 43, 45, 47, 48	1, 4, 6, 7 10, 11, 15, 16, 18, 19 20, 22, 25, 26, 28, 29 30, 31, 32, 33, 34, 35, 36, 37, 38 41, 42, 43, 45, 46, 47, 49 50	3, 7, 8 11, 15, 17 25, 29 33, 34, 37 42, 48	1 14, 17, 18 20, 28	1, 8 14, 17, 18 24 30, 34, 36 41	2, 4, 5, 7, 8 14, 16 21, 22, 24, 27 30, 34, 35, 36, 37, 38, 39 40, 47, 48
50±	7, 10 13, 15, 17 20	1 17	4, 5, 7 25, 28	1, 2, 3, 4, 5 11, 13, 17 23	8 14 21, 23, 24, 27	12, 14, 16, 18, 19 20, 24	4, 7, 8 11, 12, 13, 15, 16 23, 24, 26	6, 7 16, 19 21	1, 6 11, 12, 13, 15, 19 23, 29

37, 38	38	38, 39	34, 35, 36, 38	44	30, 35, 39	30, 32, 35	31	31, 33
40, 43, 44, 45, 47, 48	40, 48	40, 45, 46	40, 41, 46, 49 50		45, 47	41, 49	44, 45	44, 46
								50
1, 2, 5 12, 14, 16, 18	2, 5 14, 16, 18	2 13, 16, 17	5, 7 12, 16	2, 5 12, 13, 17	1, 2, 4, 5, 6 10, 13	2, 5, 6 10, 19	2, 3, 4, 5, 9 10, 11, 12, 13, 15	3 10, 17, 18
21, 22, 24, 25, 27, 28	25, 26, 27, 28	20, 21, 22, 27	22, 25, 26		21, 22, 23, 26, 27, 28	21, 22, 25, 27, 29	20, 22, 23, 25, 26, 27, 28, 29	20, 25, 26, 28
39	39		31	39	31, 32, 36, 38	31, 33, 34, 36, 37, 38, 39	32, 33, 35, 37, 38, 39	32
41, 46, 49	41, 44, 45, 46, 49	41, 47, 49	44	40	40, 41, 43, 44, 46, 49	40, 42, 43, 44, 45, 46, 47	40, 42, 43, 46, 47, 48, 49	41, 42, 43, 45, 49
					50	50		
M†	M	M	M	M	M	M	M	M

Left row brackets: 50± ; 0 to 33

* Absorption with homologous strain not always carried out, but where tested always 100 per cent.

† M = Meningococcus.

group. In the light of these findings we have concluded that this strain 42 is as nearly representative of the entire gonococcus group as any which we have encountered. This conclusion is substantiated to a considerable extent by the complement fixation tests reported in a following section.

Strains 7 and 18 show only partial relationships to those used in producing the five preceding sera; strain 7 absorbed strongly the homologous agglutinins from sera 218 and 106, partially those of 217 and 104, and none from 225; strain 18 absorbed completely or nearly so from 104, 106 and 225 sera, and not at all from 217 and 218. Sera produced with each of these two strains (7 and 18) indicated also that their agglutinogenic constituents differ markedly from those of strains 8, 15, 29, 33 and 42 in that their homologous agglutinins are absorbed strongly by relatively few of this collection of gonococcus strains. Yet, on the other hand, these two strains interabsorbed in too slight degree to warrant placing them in the same group. Strain 41 is also one which showed a close relationship to only a few of the other forty-nine gonococcus cultures. Of the seven preceding sera in table 5 this strain 41 absorbed completely only homologous agglutinins from strain 42 serum, and only nine heterologous strains caused a 75 to 100 per cent absorption of the homologous agglutinins from its own serum (223). Some of these strains absorbing strongly from strain 41 serum bring this culture into close relationship with strain 18. Strains 1, 14, 17, 18, 24 and 41, in fact, may be said to constitute a small group, but one which is not sharply separated from the main group of which strains 8 and 15 are examples.

The absorption tests with strain 5 serum (95) are of much interest in that this strain seems to serve as a connecting link between such aberrant strains as 2, 16, 21, 22 and 27, which failed largely to absorb from the preceding sera of table 5, and certain members of the main group such as 8, 30 and 34. Strain 5, as is shown in this table, is one which failed almost completely to absorb the homologous agglutinins from the eight other sera, yet the homologous agglutinins of its own serum were bound strongly by twenty of these strains. This is an example of a

phenomenon frequently observed in this study, namely, that the agglutinin-binding and the agglutinogenic properties of a given strain may exhibit apparent inconsistencies.   Meinicke, Joffe and Flemming (15) noted a somewhat similar state of affairs in their study of serological relationships of cholera vibrios and ascribed the failure of certain vibrios to bind the homologous agglutinins produced by other strains to loss of avidity of those particular receptors as indicated by *in vitro* absorption tests, but *in vivo* these receptors regain their avidity and in conjunction with the other elements constituting the specific antigen of the vibrio may produce agglutinins for the entire group.   It is conceivable, in like manner, in relation to certain of these gonococcus strains, of which strain 5 is an example, that the antigenic constituents may have become so altered as to impair their agglutinin-binding capacities and yet leave their agglutinogenic properties intact.

The absorption results with the nine sera reported in table 6 and also of two others, indicated to us that our strains could not be distributed among a number of distinct serological types which were sharply separated from one another, as is the case, for instance, as regards the first three types of pneumococci.   There would seem to be one large basal group, the members of which are closely related but not all entirely identical from an antigenic standpoint.   To this main or regular group, thirty-nine of our seventy-seven strains may be referred, namely, 3, 4, 6, 8, 9, 10, 11, 15, 19, 23, 26, 29 to 37, 40, 42, 43, 45, 47, 50 to 53, 60, 61, 63, 64, 66, 69, 70, 71, 72.   Some of these strains embody antigenic elements of a highly generalized character and are thus representative of a considerable part of the gonococcus group.   Strains 8, 15, 34 and 42 are examples of cultures possessing markedly generalized antigens.

Bordering on this main group, we have encountered sixteen strains of which the antigenic elements are more specialized but which, nevertheless, showed a close relationship to certain members of the main group.   Such strains (as examples may be cited 5, 7, 12, 13, 14, 21, 24, 38, 39, 44 of table 6) may be designated, "intermediate," in view of their position between the main group and the following variant strains.

Finally we have encountered in this collection a considerable number of strains (19) which are more definitely separated from the main group and which exhibit individual antigenic variations to a marked degree. We would designate these as "irregular strains" and place the following in this class: 1, 2, 16, 17, 18, 20, 22, 27, 28, 41, 46, 49, 55, 62, 67, 72, 75, 76, 77. Some of these strains, as 1, 17, 18, 20, and 41, as already stated, showed close enough relationships to warrant, perhaps, placing them together in a small group, although strain 1 tended later to develop the qualities of a regular strain. It is quite possible that through the use of other sera further relationships among certain of the irregular strains might have been discovered. In fact, as shown in table 6, a relationship to some one of the regular strains has been demonstrated for a number of these irregular strains, indicating that they may possess certain antigenic elements in common with the main group. We may, perhaps, conceive these variants as having undergone a rearrangement of their generalized antigens through adaptation to differing environments to such a degree that certain strains, such as 2 and 22, seem to be almost distinct serological entities. As will be indicated presently, however, it is conceivable that they all may be capable of reversion to a type approximating the original pattern.

In classifying the gonococci under the three general headings of (a) regular, (b) intermediate and (c) irregular strains, we mean to convey our impression that the antigenic constitution of these organisms is so variable and so prone to individualistic expression that the supposition of the existence of sharply defined types or groups is not warranted. This conclusion seems to us to be especially justified by the fact that the gonococcus antigen has exhibited marked tendencies to lability. This point will be discussed later in detail.

The most extensive serological study of the gonococcus group, based on the absorption of homologous agglutinins, which has been published within recent years, is that of Hermanies (13). Jötten (16) differentiated four different groups into which twenty of his twenty-seven strains apparently fell, but he does not report having used absorption methods in his classification, so our

results may not be compared with any degree of assurance with his. Hermanies' work constitutes a painstaking and close analysis of eighty-five gonococcus strains and his results are of much interest. Through the use of agglutinin absorption methods, he concluded that his eighty-five strains might be distributed among six immunological types, which he believed to be distinct and clear-cut. His group 2, consisting of thirty-six strains, was further divided into four sub-types. In his first group were included the ten so-called "Torrey strains." In a personal communication he has informed us that one set of these strains was obtained from the New York (City) Department of Health Laboratories and as our set of these strains was obtained from the same source, we may be reasonably sure that his group 1 of forty-one strains and our main group of thirty-nine regular strains correspond as, in both instances, these groups include these "Torrey strains." His second group of thirty-six cultures included strains which varied greatly in their agglutinogenic and absorptive capacities. We have not been able to detect any second large group among our series of strains, although some of our intermediate or possibly the small group of five irregular strains might be found to correspond to certain of his group 2 types. His other four types were made up of more or less isolated variants; three strains constituting his type 3, two his type 4, and one each in his types 5 and 6. It is quite possible that some of our irregular strains may correspond to these latter types.

In comparing our results with those of Dr. Hermanies and in attempting to reconcile the differences, certain considerations should be held in view, all or any of which may be accountable in some measure for the dissimilarity of our conclusions. In the first place our stock cultures have been maintained almost from the time of isolation on a semi-solid serum-free medium, whereas the Hermanies strains were carried on ascitic agar slants. It is possible that our medium is more likely to bring to light generic interrelationships than is the solid ascitic agar. Again, many of our strains were derived from widely separated geographical localities, the infection having been contracted in

a considerable number of instances in foreign countries, and as regards other cases in widely separated parts of this country. Of the foreign strains, three are from England (2, 5, 18), six from France (4, 6, 46, 66, 69, 73), two from Mexico (8, 74) and one each from Germany, Belgium, Egypt and Panama (respectively, 7, 41, 53, and 76). A number of other strains obtained from Dr. E. Finch are probably of foreign origin, but their history is not definitely known. The majority of the Hermanies strains are described as having been isolated from patients in the Cincinnati General Hospital. It is, perhaps, reasonable to suppose that such a comparatively restricted environment would yield a larger number of strains of a similar type. And, finally, our methods for conducting the absorption tests were quite different. It seems probable that we absorbed with a larger dosage of gonococci than did Hermanies and perhaps with a more uniform dosage. His technic was based on the assumption that ascitic agar slants of fairly constant area would yield a like amount of growth with each of his gonococcos strains. It has been our experience that various gonococcus strains may differ markedly in the amount of growth produced on a given lot of medium and that comparative absorption tests resting on the supposition that growth obtained from approximately equal areas of culture medium surface will prove at times misleading. Further, in the Hermanines technic absorption tests were confined to the determination of the degree of removal of the homologuos or specific agglutinins but no check was made on absorptions of the group agglutinins. It is obvious that if these are not absorbed completely they might influence the titer obtained in tests of the absorbed serum with the homologous strain. There is no statement, also, in regard to the selection of a definite end point for the agglutination reaction. In comparative work this would seem to be a matter of prime importance.

A careful study of the Hermanies tables would seem to indicate here and there some relationships between his several groups. Certain of his type 1 strains absorbed at least 50 per cent of the specific agglutinins from a type 5 serum and also a type 4 strain apparently absorbed 75 per cent from type 5 serum. In his

second article (14) table 6, there is an indication that three type 1 strains and also the type 4, 5 and 6 strains removed at least 50 per cent of specific agglutinins from a type 2(b) serum; in the same article, table 8, six other type 1 strains absorbed 50 per cent or over of type 2(a) agglutinin from a type 2(b) serum. A few other instances of apparent cross absorptions might be cited, all of which indicate that there existed at least some degree of inter-relationship between these Hermanies types. It seems to us quite likely that if antisera had been produced to a considerable number of the type 1 strains, certain ones might also have been found occupying an intermediate position between the type 1 and type 2 groups.

In his second article (14) Hermanies has analyzed his thirty-five type 2 strains and demonstrates that they may be divided into four fairly distinct and characteristic sub-types—the a, b, c and d races. The matter was futher complicated by the finding that the individual strains of these several races differ more or less among themselves in the relative distribution of these several agglutinogens. There exists, then, in this group a very complex state of relationships. The point, however, which we wish to emphasize particularly at this place is that these several agglutinogenic components, according to Hermanies' findings, may change in relative amount under cultivation; for example, as regards his strain 66, he found that after a period of cultivation the antigenic component designated as b had become much reduced with a coincident augmentation of the so-called x moiety. Other similar instances of marked lability of the antigenic constituents of this group are noted in his article.

Very recently Cook and Stafford (31) have reported, as a result of an agglutinin absorption and alexin fixation study of sixteen gonococcus strains, that they were not able to define any groups, absorption of agglutinins having taken place without uniformity. The technic employed in the absorptions was essentially that given by Hermanies.

In 1907 one of us (1) determined that six of the ten gonococcus strains studied might be distributed through agglutinin absorption experiments among three groups which were apparently

serologically independent of one another. In reëxamination of five of these strains (one was not available) we were much surprised to find that they had become very closely related as

TABLE 7

Experiment to show the changes in absorptive capacity which have occurred in certain "Torrey strains" after fourteen years cultivation. Set 1 = strains obtained from the New York City Health Department Laboratories. Set 2 = strains obtained from H. K. Mulford Company Laboratories. Set 3 = strains obtained from Parke, Davis and Company Laboratories. Test serum for 1921 absorptions immune to strain 36 (original G) of set 1. This serum was absorbed with each of the strains of each set and then tested with the homologous strain. Titer, 3000. Absorbed at 1:100

"TORREY STRAINS" (ABSORBING STRAINS)	ABSORPTIONS OF 1907		ABSORPTIONS OF 1921 WITH					
			Set 1 strains		Set 2 strains		Set 3 strains	
	Titer with unabsorbed serum	Titer homologous strain with absorbed serum	Titer with unabsorbed serum	Titer homologous strain with absorbed serum	Titer with unabsorbed serum	Titer homologous strain with absorbed serum	Titer with unabsorbed serum	Titer homologous strain with absorbed serum
A	800	2000 (0)*	2000	<200 (100)	1000	1000 (66)	1000	500 (84)
B	400	Not tested	2000	200 (94)	500	200 (94)	2000	200 (94)
C	800	2000 (0)	3000	<200 (100)	2000	200 (94)	2000	1000 (66)
G	2000†	—	3000	<200· (100)	4000	500 (84)	200	500 (84)
H	2000	800 (60)	—	—	—	—	1000	1000 (66)

* Figures in parenthesis indicate percentage absorptions of homologous agglutinins.
† Homologous.

regards their antigenic constituents. In table 7 there is presented a comparison of the absorption results with strain G (36) serum in 1907 and 1921. It may be observed that in 1907, strains A (strain B was closely related to A) and C absorbed none of the specific agglutinins from strain G serum, but strain H

absorbed 60 per cent. In 1921, however, we find that three sets of these strains obtained from three independent laboratories (H was available from only one source) had approached a common type in that all the A, B and C strains now absorbed from two-thirds to all of the homologous agglutinins from a strain G serum. It is of interest to note that, whereas a variety of media had been used in the cultivation of sets 1 and 2 during this fourteen-year period, set 3 had been maintained on an ascitic agar medium of uniform character.[4]

Other similar experiments in which a strain B (30) serum was absorbed with these three sets of cultures have given similar results, in that strains C and G, which in 1907 appeared quite distinct from strain B serologically, now absorb very strongly the specific agglutinins for strain B. Hermanies has placed all of the "Torrey strains" received from two sources in his type 1 group, although six of the ten strains received from another source constitute his c race of type 2. We also have found nine of these strains (A, 29; B, 30; C, 31; L, 32; 0, 33; S, 34; Q, 35; G, 36) sufficiently alike serologically to warrant placing them together in our group of regular strains. It is evident, then, that gonococcus strains which at the time of isolation are apparently distinct serological entities or belong to particular groups, may tend after a period of cultivation to assume a common generalized antigenic condition. We are also convinced that antigenic lability should not be postulated as confined to one particular group or type, as assumed by Hermanies, but is rather a generic characteristic shared in varying degree by all gonococcic strains.

Absorptions with twelve selected strains

Our inability to demonstrate the existence of distinct and stable types in the gonococcus family led us to approach the problem of serological relationships among these organisms from a different standpoint, namely, the application of absorption tests to a large number of different strain sera with a selected set of twelve

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[4] Set 2 was composed of strains which had been received from the New York Health Department Laboratory two to eight years previously.

strains in order to single out one or more strains of such a generalized character as to be fairly representative of the whole gonococcus group. An advantage pertaining to making absorption tests with such a limited number of strains lies in the fact that an experiment with a given serum may be carried out in a single day and thus under more uniform conditions than when the tests are extended over several days as is necessary with a large collection of strains.

These twelve gonococcus strains were in some measure representative of the principal serological variants encountered among the first fifty strains examined. The twenty-seven monovalent sera which were absorbed, were immune to strains representing a still wider range of variation. In table 8 are collated the results obtained in this comparative series of absorptions. The figures under each serum represent the percentage of absorption of the homologous agglutinins which was effected by each of the several selected strains. In the case of a few sera an absorption was not carried out with the homologous strain but it may be assumed that 90 to 100 per cent of the agglutinins would have been removed. With reference to anti-sera to strain 5 and also to 29, the results with two sera produced at different times are given in order to illustrate fluctuations in absorptive capacities which were observed in certain strains—such as 1, 7, 18, and 25—under conditions of artificial cultivation. These experiments were all conducted and controlled in the manner illustrated in table 5, but for our present purposes it is sufficient to report the data in regard to the percentage of absorption of the homologous agglutinins.

Table 8 shows clearly that there exists a diversity in the range of the absorptive capacities of these selected strains. In order, however, that these differences may be readily evaluated, a graph is presented giving the percentage absorption value of each strain. In computing these percentage values, a score of 2 points was given for a practically complete absorption of the homologous agglutinins (75 to 100 per cent); for a partial absorption (about 50 per cent) 1 point, and a slight absorption (not exceeding 33 per cent) was counted as negative. On this

TABLE 8

Absorptions of 27 gonococcic sera with 12 selected gonococcus strains and 1 meningococcus strain. The figures indicate the percentage absorption of homologous agglutinins

GONOCOCCIC SERA; SERUM STRAIN NUMBER FIRST AND RABBIT NUMBER IN PARENTHESES

Column headings (strain number (rabbit number)): 1 (219), 3 (247), 5 (251), 5 (121), 8 (225), 11 (118), 13 (96), 14 (103), 15 (104), 18 (122), 19 (119), 25 (125), 27 (81), 28 (82), 29 (217), 29 (236), 30 (236), 33 (218), 34 (116), 36 (127), 38 (106), 41 (235), 42 (106), 46 (83), 48 (227), 49 (129), 53 (107), 56 (108), 64 (239)

Row labels: 1, 7, 11, 15, 18, 19, 25, 30, 34, 38, 41, 49, Meningococcus

Homologous titers and absorption dilutions of above sera

	Homologous titer	Absorption dilution

337

basis, then, the highest possible score for the twenty-seven sera would be 54.

This graph shows clearly the fact that of these particular strains, 34 possesses the widest and most generalized absorptive capacities, effecting a marked absorption of the homologous agglutinins from twenty-four of the twenty-seven sera. This
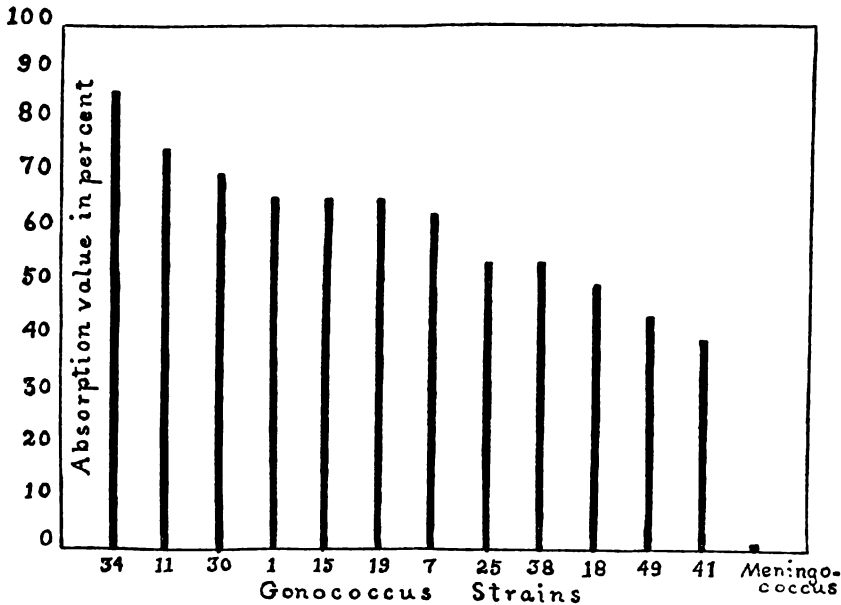
CHART 1

strain, as has already been mentioned, is one of the set obtained from the New York Health Department Laboratories under the designation "S." Strains 11 and 30 proved to be next in efficiency in absorptive capacities with the others grading off to those with the least generalized absorptive properties, namely, strains 41 and 49. We do not wish to convey the impression that strain 34 is unique among our collection of cultures in its generalized affinities for it seems highly probable that certain other strains, such as 8 and 42, might have proved quite as effective.

General observations in reference to table 8

Tolluch (17) in discussing the importance of the application of standardized agglutination methods in a study of immunological types of a given bacterial species, mentioned the desirability of using sera with as uniform a titer as possible. Other investigators, such as Gordon (18) for meningococci and Hooker (7) for the typhoid bacillus, have reported the presence of greater specificity in sera from rabbits after a few inoculations than after many; or as Gordon has expressed it, in the "first born agglutinin." Although these general principles may apply to the gonococci, it was not found feasible either to restrict the number of inoculations to a small number or to attempt to employ sera with a nearly uniform titer. This was due to the fact that the rabbits responded very unequally to inoculations with various strains. With some strains a fairly high titer could be obtained after six inoculations given in a period of two weeks, but with other strains a prolonged course of immunization was necessary before a serum could be obtained with a titer perhaps only one-half or less as high as with the former.

As has already been mentioned, relatively inagglutinable strains were encountered more frequently than was anticipated. Inagglutinability, however, does not necessarily mean the absence of agglutinogenic or agglutinin-absorbing capacities as was demonstrated by McIntosh and McQueen (19) some years ago in a study of an inagglutinable typhoid strain, and more recently in reference to typing meningococci by Mathers and Herrold (20) and others. On the other hand Bemains (21) has shown that a strain of *B. dysenteriae*, which was artificially rendered inagglutinable, also lost its agglutinogenic and agglutinin absorbing functions. It might be expected that the relatively inagglutinable strains of gonococcus would also prove poor absorbers of agglutinin, but this, although true in some instances, was not always the case; certain poorly agglutinating strains, such as 25 (table 8) absorbed agglutinins strongly and consistently. Another similar point, which has already been 'discussed, is further illustrated in table 8, namely, that the agglutinogenic and the absorptive capac-

ities of a strain may not run entirely parallel.   For example, strain 38 produced a serum from which the homologous agglutinins were absorbed in greater or less degree by all these twelve selected strains and yet strain 38 removed none of the homologous agglutinins from sera produced respectively with strains 1, 18 and 30. Other instances are provided by strains 27 and 49, both of which absorbed agglutinin poorly and were apparently unrelated to other larger groups of gonococci but which, nevertheless, produced agglutinins readily absorbable by regular types.   In determining serological relationships among the gonococci, then, it is not sufficient alone to absorb other sera with a given strain but a serum should be produced with this strain and absorbed in turn with other typical strains as it is evident that in some instances the function of binding agglutinin may have become inactivated or suppressed in some degree without affecting the agglutinogenic propensities.

Teague and McWilliams (22), in a study of the effect of spontaneous agglutination on the absorption of agglutinins, found that in the case of a typhoid strain spontaneously agglutinating sub-cultures absorbed less agglutinin than did the normal sub-cultures. They inferred from this finding that physical aggregation may play a part in effecting the amount of agglutinin absorbed by various strains.   Gonococcus strains with tendencies to spontaneous clumping have been encountered not infrequently in this work but this tendency has not seemed to interfere with agglutinin absorption, perhaps because the aggregations were more readily broken up than is the case with flagellated bacilli such as *B. typhosus*.

Vulvovaginitis strains

This series of gonococcus cultures included thirteen strains isolated from cases of vulvovaginitis in young girls.   The ages of these patients ranged from two to eleven years, with ten under six years.   These strains were studied with particular care in order to determine if they constitute a type immunologically distinct from those recovered from adult gonorrheal infections. Louise Pearce (23), from a comparative serological study of six

infant strains and nine strains from adult cases, came to the con-
clusion that there are inherent differences in the types of gonococci
causing these two classes of infections, although not to an extent
which would warrant separating them into two distinct immuno-
logical groups.   Her methods of investigation included agglutina-
tion tests without absorptions and complement fixation experi-
ments.   The results with these two methods were harmonious.
It is obvious that if the gonococcal strains recovered from these
two classes of infection could be shown to be representative of
serologically distinct types, it would clarify our ideas in regard
to their epidemiological relationship.

In table 9 the results of absorptions with sera immune to two
of these vulvovaginitis strains are reported and in table 8 the
absorptions of these sera (108 and 239) with twelve adult strains.
In table 5 are detailed further the absorptions of a serum (104)
immune to an adult strain (15) with these infant case strains.

These several cross absorptions indicate clearly that no definite
serological distinction may be drawn between infant and adult
strains.   Among these infant strains, as is demonstrated in
table 9, one encounters the same lack of homogeniety as to type
as would be observed among a similar series of strains isolated
from adult cases.   We also find among the infant strains cer-
tain ones (48, 60, 61, 63, 64) which absorbed strongly the homol-
ogous agglutinins from an adult strain serum (table 5) and also
others (55, 56, 57, 62, 75) which removed little or none of such
agglutinins from this adult, regular strain serum.   One fairly
definite characteristic, however, was noted in the study of these
infant strains and that was that their agglutinogenic potential-
ities were not strong.   No serum with a titer above 1:800 was
obtained with any of the four strains employed in rabbit immuni-
zations.   With one strain (48) the titer could not be forced
above 1:250 in spite of a prolonged series of inoculations of large
dosage.   It should be noted, however, that the production of
low titer sera was not confined to infant strains as certain adult
strains, especially of the irregular type, exhibited the same ten-
dency, but it appeared to be more of a distinguishing trait of
the infant strains.   These infant strains also, as a class, tended

to absorb agglutinins rather less strongly than most of the adult strains. This weakness in antigenic properties may be a factor in determining the mild yet chronic course which most of

TABLE 9

Two sera immune to vulvovaginitis strains (children cases) 64 and 56, absorbed with other strains from the same type of cases.

STRAINS	TITER WITH UNABSORBED SERUM	TITER ABSORBING STRAINS WITH ABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH UNABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH ABSORBED SERUM	PERCENTAGE ABSORPTION OF SPECIFIC AGGLUTININS

STRAINS	TITER WITH UNABSORBED SERUM	TITER ABSORBING STRAINS WITH ABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH UNABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH ABSORBED SERUM	PERCENTAGE ABSORPTION OF SPECIFIC AGGLUTININS
Strain 64 serum, titer 800; absorbed 1:25					
55	50	<50	800	100	88
56	<50	<50	800	800	0
57	<50	<50	800	400	50
58	200	<50	800	200	75
59	400	50	800	100	88
60	400	100	800	200	75
61	200	50	800	100	88
62	100	<50	800	400	50
63	800	100	800	800	0
64*	800	100	800	100	88
65	100	<50	800	800	0
75	400	<50	800	400	50
Meningococcus	<50	<50	800	800	0
Strain 56 serum, titer 500; absorbed at 1:25					
48	100	<50	500	50	90
54	750	100	500	250	50
56*	500		500		
58	500	100	500	100	80
60	1000	<50	500	250	50
61	1000	<50	500	250	50
62	100	<50	500	250	50
64	750	100	500	100	80
Meningococcus	<50	<50	500	500	0

* Homologous.

these cases run in that insufficient stimulus is provided for the production of an adequate amount of bactericidal antibody on the part of the host.

It should be observed that the majority of these vulvovaginitis strains were isolated from patients under treatment at a clinic

(Vanderbilt Clinic, through the courtesy of Dr. B. Wallace Hamilton) and it was thus entirely unlikely that the infection could have been received in any instances from a common source. On the other hand, if such strains are isolated from cases occurring in an institution, there is always a possibility that a single strain may have been spread through contact and, accordingly, a study of such cultures might give an unwarranted impression of serological homogeniety as existing among gonococci causing these vulvovaginitis infections.

DISCUSSION

Within recent years much time and effort has been devoted by various investigators to the detection and formulation of distinct immunological types among different species of pathogenic bacteria, the members of which are culturally identical or nearly so. Without doubt these efforts, in some instances, have served a very useful purpose. There is, however, the lurking hazard in extending this method of grouping to other bacterial species that one may depart widely from a natural system of classification. A type or group, if established on a rational basis, should represent a distinct serological entity. The antibodies of a serum produced with a member of a given type should be absorbed completely, or nearly so, by all strains referred to the same type but should not be affected by strains assigned to a different type. Or, as Eastwood (24) has expressed it in an illuminating discussion of this question, "if strains belonging to the species fall into distinct groups without cross-division, when tested by a number of monovalent sera, grouping is indicated; but if such tests produce marked cross-division, grouping is not justifiable." To quote further, "where each main group is so elastic that its margin of separation from the others is small, one begins to raise the question whether the adopted system of grouping has turned out to be artificial and arbitrary, and whether the species under consideration is really amenable to subgrouping."

In these remarks, Eastwood was referring particularly to certain proposed groupings of the meningococci. Dopter's orig-

inal division of the meningococci into the true meningococci and the parameningococci was a natural one in that we have here two groups which are generally recognized as being serologically distinct, but in the further division of the meningococci into four groups in accordance with Gordon's classification (18), two groups have been added which, perhaps, are not founded on as sound a basis. In fact, a considerable number of investigators of the meningococci have reported that the Gordon group III is not sharply separated from I, nor IV from II; affinities which Gordon, himself, has recognized. Griffiths (25) and also Scott (26), from a study of spinal and nasopharyngeal strains derived from much the same sources as those of Gordon, both concluded that only two main groups should be recognized; a conclusion which Eastwood has considered justifiable. Griffiths has also inferred that "the two groups are not fixed types but may be further sub-divided by means of absorption experiments into sub-groups which are probably 'centers of variation' of the different stages of evolution of the meninogococcus antigens."

It is the opinion of the writers that there is much less justification for the formulation of distinct groups in the gonococcus than in the meningococcus family. Among the gonococci there are no two groups as sharply separated as the normal meningococci and the parameningococci. In fact, if agglutinin absorption and agglutinogenic properties of *each* strain are taken into consideration, as they really should be in an attempted formulation of types, we would find so many strains exhibiting individual immunological variations and also inter-relationships between what might, at first sight, be considered representatives of distinct types that the line of demarkation between the proposed groups would tend to become completely obliterated. We have, accordingly, on the basis of the evidence already presented, distributed our gonococcus strains under three general headings, namely, regular strains, the most generalized as regards antigenic properties, intermediate strains, which are quite closely related to certain of the regular types, and the irregular strains which exhibit marked individualistic variations. In employing these general terms we are adopting a mode of classification similar

to one which has been extensively applied to the meningococci, but in view of our findings, especially as regards the marked antigenic lability inherent in the gonococcus group, we feel that it is as definite a one as the conditions justify.

This phenomenon of antigenic lability, as exhibited by the gonococcus, is most interesting. As has been mentioned, Hermanies noted a marked degree of lability on the part of certain members of his type 2 group. During the course of our experiments we have observed numerous instances in which the antigenic constitution of strains have undergone some change under cultivation. By way of example reference may be made to the results obtained with two sera (217 and 236, table 8) immune to strain 29. An interval of over a year occurred between the times of production of these sera against a strain which had become stabilized. It may be noted, however, that strain 1 has, during that interval developed affinities very similar to the serum strain 29, a member of the regular group, although soon after isolation this strain 1 showed a close relationship to strain 41, a member of the irregular group (table 8). Strain 25, also, during this period has developed evidence of a close relationship to this serum strain, and strain 7 has passed from an intermediate position to a close affinity to the regular group. These are only a few instances, among many, in which there has occurred a change in antigenic responses to various sera. Beside such changes in these comparatively recent strains, we have the still more striking evidence of antigenic lability as presented by the changes which have occurred in the "Torrey strains" during the fourteen years of cultivation (table 7). In the face of such a tendency to mutation in antigenic constitution on the part of gonococci, one would have no assurance that strains selected as type representatives might not change entirely in character after a period of cultivation. We believe, in fact, that the whole tendency under conditions of artificial culture is for reversion to our regular group and that a strain having attained that disposition of its antigenic components remains in a comparatively stable condition.

Changes in type under cultivation have also been noted by

various observers as occurring in certain strains of the meningo-cocci. Griffiths, in fact, in view of the variations in antigenic characteristics observed during sub-cultivation concluded that "meningococcus antigens are not precisely fixed or stable sub-stances, but are liable to modification under the influence of environment." He believed these changes might take one or the other of two directions; toward either increased or diminished complexity of the structure of the receptor apparatus, which would mean an increase or diminution in the range of binding capacity. Scott has likewise reported temporary variations in agglutination between different sub-cultures of the same strain of meningococcus, and also variability in the absorptive power of one strain. Both of these phenomena, however, he considered due to the presence of two varieties of cocci of differing sensitivity to agglutinin and absorptive powers within a single strain with sometimes one and sometimes the other predominating and not to antigenic changes within the substance of the cocci. These peculiarities, however, in the absorption of agglutinin and also the large number of serological varieties which he encountered, caused him to doubt the practical value of this mode of classi-fication in defining types among the meningococci. Walker (27) has concluded in connection with a discussion of the signi-ficance of meningococcic types that "fixity of these (Gordon's) is not proved." Butterfield and Neill (9), although believing Gordon's type strains to be stable and finding sera prepared with them very effective in the classification of their series of meningococci, reported that certain strains during a year's time apparently changed from one type to another. A very interesting observation in this connection is that of Eberson (28) on the effect of ultra-violet rays on the antigenic properties of the meningococci. He found that by exposure to these rays, regular and irregular meningococci could be so altered that they developed the property of producing mutual agglutinins, but parameningococci could not be so altered. He further concluded from similar tests that regular types of meningococci include with-in their protein molecules the elements of the parameningococci but that the latter possesses no agglutinogenic radicle common to the regular and thus constitutes a distinct type or species.

In attempts to explain the development of serological variants among certain groups of bacteria, a number of different hypotheses have been offered. The theory of Meincke and his colleagues of the loss of avidity on the part of certain receptors of the cholera vibrio has already been mentioned. Eastwood (24) has suggested that a more reasonable explanation of these changes in antigenic behavior lies in the conception that antigen is "a chemical substance which may exist in one or other of several different chemico-physical phases, demonstrable *in vitro*. And the same conception would apply to antibody." He further suggests that these various chemico-physical phases are closely related to changes in stereo-chemical structure of the molecules of the bacterial protoplasm. This theory, of course, is at present highly speculative, but it may find confirmation through later advances in physical chemistry. Hermanies, in a discussion of the origin of the sub-groups and races which he encountered in his group 2 of gonococci, proposes the theory that his four types were originally derived from a common type containing alone the agglutinogen x, but under different environmental conditions this x moiety has tended to retrogress and has been replaced in greater or less degree by the antigenic elements a, b, c and d, according to the particular race to which a given strain is related. This process, he suggests, may be carried so far that the original x element becomes lost and a new species characterized by one or other of the four acquired antigenic elements may arise. The new species would seem to be fixed in type because, as the antigenic element x has been entirely eliminated, there is no possibility of reversion to that original type. In view, however, of our experimental results it seems to us that this theory assumes a greater degree of acquired antigenic stability than is exhibited by the gonococcus group. It is our opinion that as a result of adaptations of gonococcal strains to different environments, including the defensive agencies of the host, there have arisen varied molecular configurations or physical-chemical phases—to use Eastwood's term—of the same specific basal substance, none of which, however, attain a state of complete stability and thus do not give rise to new fixed types. Further-

more, as none of the original elements have been lost or eliminated, there may occur in time following exposure to an environment of fairly uniform type, such as is presented by a culture medium, a molecular redistribution of the antigenic substance until a pattern resembling that characteristic of our regular and generalized group is attained.

Griffiths (25) has suggested that enhanced virulence and invasive powers of the meningococcus may be correlated with increased complexity of the receptor apparatus; that is with an increase in range of combining capacity. From this point of view our most complex gonococcic strains are those designated as regular, with the intermediate next in order and the irregular types exhibiting the least degree of complexity. Although the majority of our strains were isolated from more or less acute cases of urethritis and accordingly their invasive propensities could not be evaluated, there were eleven strains in the series recovered from cases showing complications. Among these there was one case of ophthalmia, three of chronic prostatitis, one epididymitis, four joint infections and two septicaemias. Ten of the eleven strains isolated from these cases showing complications were of the regular or intermediate type. Although the number of these cases is too few for a definite conclusion, the tentative suggestion may be made that the irregular variant strains are less likely to give rise to complications than are those resembling the regular types. Jötten (16) found that his more virulent strains fell into two of his four groups, but as to whether the members of these two groups are serologically related to our regular types, we, of course, can offer no opinion.

COMPLEMENT FIXATION

In these complement fixation experiments with the gonococcus our purpose has been not so much to confirm the conclusions derived from the agglutination work in regard to the serological relationships of our strains as to apply the facts disclosed to the selection of strains most useful as antigens for the diagnostic complement fixation test. Incidentally, however, we have noted a marked degree of correspondence between the strain affinities revealed through these two methods.

One of the great obstacles to the general use of this test in the past has been the difficulties associated with the preparation of the antigen, especially as the delicate nature and apparent fastidious cultural requirements of the gonococcus have made their maintenance a troublesome matter and also because it has seemed necessary to employ a considerable number of strains in preparing the antigens. Commercial distributers of biological products, also, have been unsuccessful in supplying this antigen in an entirely satisfactory way because of its tendency to develop anti-complementary properties. It would seem, then, that if one or two readily cultivatable, representative strains should prove to be as efficacious as antigens as the combinations of the ten or more strains, which have been used generally heretofore, the test would be much simplified and hence more generally available.

<div align="center">TECHNIC</div>

Antigen. The following serum-free solid medium has been used for growing the cultures for antigens. This medium has a growth accessory principle and is prepared according to the Huntoon (4) method, although the formula has been modified. It has generally yielded a luxuriant growth with the gonococcus strains employed.

Salt-free, 1.5 per cent peptone, "vitamine" agar. Five hundred grams of fresh chopped beef heart, free from fat, one whole egg and 1 liter of distilled water are placed in a double boiler over a free flame and the temperature is maintained at 60°C. with constant stirring for five minutes. Fifteen grams of peptone (Difco) and 18 grams of flaked agar are now added and the temperature raised until the medium assumes a brownish color. It is then made slightly alkaline to litmus (with a 10 per cent sodium carbonate solution) and is transferred to a flask, or better to a coffee pot, and heated in the Arnold sterilizer at 100°C. for two hours. The medium may now be cleared through centrifuging or by filtering through glass wool; if the latter procedure is used, the meat residue should be deposited on the glass wool in a funnel and the fluid portion allowed to perculate through; several times, if necessary. No cloth, cotton or other absorbent material

should be allowed to come in contact with it. After clarification, 2 per cent glycerine is added, the reaction is adjusted to pH 7.2, and the medium reheated. It is then distributed in potato tubes, sterilized in the Arnold and slanted. The slants should be laid down not longer than the day before they are to be used.

The large slants of this medium should be seeded from twenty-four hour growths on the same medium. They are then incubated for from twenty-four to forty-eight hours. In preparing the antigen the method of M. A. Wilson (29) was followed. The growth was well washed with 50 per cent and then 95 per cent alcohol, being allowed to stand one-half hour with each in a water bath at 37°C., with frequent shaking. After centrifuging, the sediment is covered with ether and allowed to stand at room temperature for one-half hour with frequent stirring. It is then centrifuged, the ether decanted off and the sediment placed over night in a dark place. The dry powder is then emusified in 0.85 per cent saline to the standard density and the proper dosage determined. It should then be heated at 80°C. for one hour, although in our experiments this heating was omitted as the antigens were prepared freshly at frequent intervals. The antigen may be preserved with 0.1 per cent phenol. Antigen prepared in this way yielded results superior to those obtained with filtered autolysates or with an antigen prepared according to Thomson's (30) method.

Gonococcic sera. Most of the immune sera were the same as those used in the agglutination experiments and, for the most part, had been preserved for a longer period of time. They were inactivated shortly before each test was performed.

Complement. The sera from three or four guinea-pigs were pooled and preserved by freezing. There was usually little or no loss in potency for four or five days. Only active complement, fixable by gonococcus, was used.

Hemolytic system. The anti-sheep system was used. One hemolytic unit of complement and two hemolytic units of amboceptor were employed. The complement was titrated immediately before the test and the amboceptor at frequent intervals. The complement unit was read at the end of thirty

minutes in the water-bath at 37°C. Washed sheep corpuscles in a 2.5 per cent suspension were used, and the amboceptor and cells added separately.

The test. The total volume of the mixed reagents was adjusted to 2.5 cc. One unit of antigen was used and this was determined by titration with a polyvalent gonococcic serum or one with generalized affinities. This unit was the smallest amount of antigen which caused complete fixation of complement with 0.1 cc. of a 1:100 dilution of the gonococcic test serum. The antigen was diluted so that this unit was contained in 0.1 cc. This unit, of course, was always less than one-half the anti-complementary dose and generally much less. This titration was usually made just before the fixation tests were started. Controls were always prepared for the hemolytic system, for anti-complementary action of the immune sera and also the antigen, and also for the stability of the sheep cells. The tests were incubated at 37°C. for the usual periods and readings made at the end of one hour-incubation of the completed test and also after the tubes had stood over-night in the ice-chest. The last reading is the one which is recorded. In accordance with the usual custom, the symbol 4 indicates complete fixation; 3, almost complete fixation; 2, the hemolysis of about one-half the cells; and 1, only a slight degree of fixation.

COMPARATIVE TESTS

In table 10 are recorded the results of comparative tests of the fixation with twenty-seven monovalent gonococcic sera, with a polyvalent sera which was also used in titrating the antigens, and with a meningococcic serum. The four antigens employed in these tests consisted of the following single strains or combinations of several strains: First, strain 34; second, strains 15, 18, 34 and 41; third the ten "Torrey strains;" fourth, strain 42. In order to give full comparative value to the results, the four antigens were always tested on a given serum simultaneously and thus under exactly similar conditions. The object, as stated, was to determine whether such generalized strains as 34 and 42 yielded as good results as combinations of several

TABLE 10

Complement fixation with 27 monovalent and 1 polyvalent gonococcic sera and 1 meningococcic serum, using the following four gonococcic antigens: (1) Strain 34; (2) Strains 15, 18, 34, 41; (3) Ten "Torrey strains"; (4) Strain 42

SERUM STRAINS	ANTI-GENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
2 (246)	1	4	3	3	3	2	1	—	—	—
	2	3	3	2	2	1	1	—	—	—
	3	4	3	3	2	1	1	—	—	—
	4	3	3	3	3	2	2	—	—	—
5 (121)	1	4	4	4	4	4	3	3	2	1
	2	4	4	4	4	4	3	2	—	—
	3	4	4	4	4	3	3	3	2	—
	4	4	3	3	2	1	—	—	—	—
7 (231)	1	4	4	3	2	1	—	—	—	—
	2	4	4	4	4	3	2	2	1	—
	3	3	3	3	3	2	1	—	—	—
	4	4	4	4	4	3	3	2	1	—
8 (243)	1	4	4	4	4	3	3	3	1	1
	2	4	4	4	3	3	3	2	1	—
	3	4	4	3	3	3	—	—	—	—
	4	4	4	4	3	3	3	2	—	—
11 (118)	1	4	4	4	4	3	3	2	1	—
	2	3	3	3	3	2	1	1	1	—
	3	3	3	3	3	2	1	1	—	—
	4	4	4	4	4	3	2	2	—	—
14 (103)	1	4	4	3	3	3	2	1	—	—
	2	4	4	4	3	2	1	1	—	—
	3	4	4	3	3	3	2	2	1	1
	4	4	4	4	4	4	4	3	2	1
15 (104)	1	4	4	4	4	4	4	4	3	3
	2	4	4	4	4	4	4	3	3	1
	3	4	4	4	4	4	3	3	3	3
	4	4	4	4	4	4	3	3	2	2
16 (87)	1	3	3	3	3	2	1	—	—	—
	2	3	2	1	—	—	—	—	—	—
	3	3	3	2	1	—	—	—	—	—
	4	3	3	1	—	—	—	—	—	—

TABLE 10—*Continued*

SERUM STRAINS	ANTI-GENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
18 (237)	1	3	3	1	1	1	1	1	1	1
	2	4	4	3	3	—	—	—	—	—
	3	4	4	3	3	2	2	1	1	1
	4	4	4	3	2	—	—	—	—	—
19 (119)	1	4	3	3	3	2	2	1	1	—
	2	4	4	3	3	1	—	—	—	—
	3	4	4	3	3	2	—	—	—	—
	4	4	4	4	3	2	—	—	—	—
25 (125)	1	4	4	4	4	3	3	3	2	2
	2	4	4	4	4	3	3	2	2	1
	3	4	4	4	4	3	3	3	2	—
	4	4	4	4	4	3	3	3	—	—
27 (81)	1	4	4	2	—	—	—	—	—	—
	2	3	3	1	—	—	—	—	—	—
	3	3	3	—	—	—	—	—	—	—
	4	3	3	3	3	2	2	—	—	—
28 (82)	1	1	1	1	1	1	—	—	—	—
	2	3	2	2	—	—	—	—	—	—
	3	4	—	1	1	—	—	—	—	—
	4	4	3	3	2	2	—	—	—	—
29 (236)	1	4	4	4	3	2	1	—	—	—
	2	4	3	3	3	1	1	1	1	—
	3	4	3	3	3	3	2	1	1	—
	4	4	4	4	3	2	1	—	—	—
30 (117)	1	4	4	4	4	4	4	3	—	—
	2	4	3	3	3	3	2	2	1	—
	3	4	4	3	3	3	2	2	1	—
	4	4	4	4	4	3	—	—	—	—
34 (128)	1	4	4	4	4	4	4	4	4	4
	2	4	4	4	4	4	1	—	—	—
	3	4	4	4	4	3	2	1	—	—
	4	4	4	4	3	1	—	—	—	—
36 (127)	1	4	4	3	3	2	1	—	—	—
	2	4	4	3	2	2	2	1	—	—
	3	4	4	4	3	3	2	2	1	—
	4	4	4	4	4	3	—	—	—	—

TABLE 10—*Continued*

SERUM STRAINS	ANTI-GENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
38 (105)	1	4	4	4	4	3	3	3	3	2
	2	4	4	3	3	3	2	—	—	—
	3	4	4	4	4	4	3	2	1	—
	4	4	3	3	3	3	3	1	—	—
41 (238)	1	4	3	3	3	2	1	—	—	—
	2	4	4	4	4	3	3	2	2	—
	3	4	4	3	3	2	2	1	—	—
	4	4	4	3	3	2	2	2	—	—
42 (244)	1	3	3	3	2	—	—	—	—	—
	2	4	3	3	2	—	—	—	—	—
	3	4	4	3	3	1	—	—	—	—
	4	3	3	3	3	3	2	2	—	—
49 (129)	1	4	4	3	3	2	—	—	—	—
	2	4	3	2	2	—	—	—	—	—
	3	4	3	3	3	2	—	—	—	—
	4	4	4	4	3	—	—	—	—	—
53 (107)	1	1	4	4	4	4	4	3	2	1
	2	2	4	4	4	3	3	3	2	2
	3	2	4	4	3	3	3	3	3	3
	4	—	4	4	4	4	4	3	3	1
56 (108)	1	4	4	4	3	3	3	3	3	1
	2	3	3	3	3	2	2	1	1	1
	3	4	4	4	3	—	—	—	—	—
	4	4	4	3	2	1	—	—	—	—
62 (234)	1	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—
	4	3	3	2	2	2	2	—	—	—
64 (239)	1	4	4	4	4	4	4	3	3	3
	2	3	3	3	3	2	2	1	1	1
	3	3	2	1	—	—	—	—	—	—
	4	4	4	4	4	3	3	2	2	—

TABLE 10—*Concluded*

SERUM STRAINS	ANTI-GENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
74 (241)	1	4	4	2	1	1	1	1	1	—
	2	1	2	—	—	—	—	—	—	—
	3	3	3	3	1	—	—	—	—	—
	4	4	3	3	3	—	—	—	—	—
77 (245)	1	4	3	—	—	—	—	—	—	—
	2	4	3	—	—	—	—	—	—	—
	3	4	2	1	—	—	—	—	—	—
	4	4	3	1	—	—	—	—	—	—
Polyvalent 221	1	4	4	4	4	4	4	3	3	2
	2	4	4	4	4	4	3	3	3	2
	3	4	4	4	4	4	4	3	3	3
	4	4	4	4	4	4	4	3	3	3
Meningo-coccus (123)	1	3	2	1	—	—	—	—	—	—
	2	2	—	—	—	—	—	—	—	—
	3	3	—	—	—	—	—	—	—	—
	4	3	2	—	—	—	—	—	—	—

strains. The second combination was selected as these four strains seemed at the time to be fairly representative in their affinities of our series of gonococcus cultures. As the results show, however, they failed to cover the variations exhibited by our collection of strains.

The results in table 10 substantiate fully the conclusion which we reached as the basis of our agglutination experiments that our strains 34 and 42 are both markedly generalized in their affinities. Further it appears that an antigen prepared with either one of them yields on the average *better* results than those obtained with the combination of the ten "Torrey strains" (antigen 3) and also than with the four strains combined in antigen 2, although the latter was expected to exhibit a wider range of affinities. In the case of no serum was fixation obtained with multiple strain antigen 3 in which the single strain antigens 1 or 4 failed; on the other hand with nine sera stronger fixation occurred with antigen 1 than with antigen 3, and with eleven sera stronger with 4 than with 3.

In view of the above results, if a single antigen is employed for diagnostic tests, we would advise using either strain 34 or 42 alone, or perhaps in combination. It seems very questionable if better results would be obtained by combining a large number of regular and irregular strains in an attempt to produce a polyvalent antigen. In the first place it is questionable if such an antigen covering all the probable variants could be prepared; and in the second place when a large number of strains each with more or less limited affinities and representative of an equal number of so-called types are combined in a single antigen, then the antigenic elements for each of these types becomes so diluted that, in the dosage permissible, the effectiveness for each type would be greatly curtailed. Perhaps the best procedure would be to use two separate antigens in each test; one prepared with one or two representative strains with generalized affinities and good combining qualities, and the other with selected irregular strains.

Selected strains for diagnostic and therapeutic applications

For the preparation of a stock vaccine for use in cases for which an autogenous vaccine is not available, we would recommend a combination of our strains 15, 34 and 41. It seems probable, also, that these strains would serve as well as any in connection with the production of a curative serum.

Strains 15 and 41 in combination may be used to advantage in the production of an agglutinating serum with wide affinities which might prove helpful in the identification of gonococci.

For the preparation of an antigen for complement fixation we advise the use of our strains 34 and 42. This antigen should exhibit generalized and strong fixation properties and prove effective except in the case of the relatively few infections due to certain irregular strains of the gonococcus. With sera giving negative results with this antigen it would seem advisable to conduct a test with an antigen prepared from a combination of irregular strains such as 16, 22, 28, 41, 49, 62 and 77 before giving a negative report. Without doubt at times inconsistencies between the results obtained with this complement fixation test

and clinical and bacteriological findings have been due to the fact that the infecting strain was immunologically unrelated to those used in the antigen. Through the use of these two antigens we believe the chance of this occurring is considerably lessened, but even so no definite assurance may be offered that one or the other would combine with the antibodies produced by all possible variants of the gonococcus.

SUMMARY

1. An analysis by agglutinin absorption methods of the serological relationships of seventy-seven gonococcus strains, isolated from cases of acute and chronic gonorrhea and its complications, indicated that they may *not* be distributed among a number of clear-cut immunological types. These gonococcus strains were representative of those occurring in widely separated geographical localities.

2. Although this investigation has not demonstrated the existence of distinct immunological types to each of which a considerable number of gonococcus strains might be referred, it was found feasible to classify our strains under the three general headings of (*a*) regular, (*b*) intermediate and (*c*) irregular strains.

3. Evidence is submitted which indicates the existence of a marked tendency to antigenic lability on the part of the gonococcus. We believe that instability of antigenic constitution is a general characteristic of the gonococcus group and that the strains exhibiting this tendency may not logically be segregated in one particular type, as has been suggested by Hermanies.

4. Among our regular strains, we have found certain ones which are highly generalized from the antigenic standpoint, and which appear to be representative of a large part of the gonococcus group.

5. Cross absorption experiments have indicated clearly that no definite serological distinction may be drawn between strains isolated from vulvovaginitis cases in children and those from gonorrheal infections in adults.

6. A few representative strains with generalized relationships and good antigenic properties have been designated as suitable

for use in a stock vaccine, and also for the preparation of a poly-valent antiserum.

7. A marked degree of correspondence was noted between strain affinities as revealed by agglutinin absorption and complement fixation tests.

8. Two strains of gonococcus have been selected, each of which covers a large part of the group, and which may be used to advantage in preparing an antigen for complement fixation diagnostic tests. It is also suggested that a second antigen, prepared from a number of selected irregular strains, be employed in conjunction with the one prepared from the two generalized strains.

9. By the use of the simple sterilizable media, which have been described, these strains may be carried in laboratories with limited bactrieological facilities and the antigens prepared quickly, whenever required.

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CULTURAL METHODS FOR THE GONOCOCCUS *

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In connection with a serologic study [1] of the gonococcus it was necessary for us to devise methods and mediums whereby this organism might be isolated readily from gonorrheal infections, and also a large collection of strains might be maintained with a minimal risk of any of them being lost. The difficulties inherent in the cultivation of the gonococcus are evidenced by the great variety of mediums and procedures which have been recommended for this purpose, especially within recent years. At present there seems to be no consensus of opinion as regards the best methods to use. In the following section certain simple mediums are described which have given good results consistently in connection with the various purposes specified. It may be remarked that through the use of a sterilizable medium of proper reaction hardly greater difficulty has been encountered in maintaining a large collection of gonococcus strains than would be associated with carrying a like number of typhoid strains.

In another article [2] are reported the results obtained through the application of these methods to the isolation of gonococcus strains from cases of mild chronic gonorrhea in women, together with a comparative study of the relative value of the cultural, complement-fixation and smear methods of diagnosis of such cases.

MEDIUMS

As a matter of convenience, the mode of preparation of certain of the mediums employed in these experiments will be described here, leaving the discussion of their uses and particular advantages to subsequent sections.

A. Ascitic-veal-urine-glycerol-agar. This plating medium, especially when combined with the dye, iodin-green, was found effective in the isolation of the gonococcus. The Thalmann [3] method of preparation has been followed to some extent, but the formula has been materially modified.

Place 1,250 gm. of fresh, chopped, fat-free veal and 2 liters of distilled water in a pot and bring slowly to a boil, allowing it to simmer for 20 minutes, with occasional stirring. Strain through cotton flannel, cool and remove the fat. Place in a double boiler over a saturated brine bath and raise the temperature to about 60 C. Add 20 gm. of peptone (Difco used), 40 c c normal, fresh urine, 10 gm. NaCl, 40 c c glycerol and 36 gm. flaked agar. Allow this to boil for 45 minutes and then adjust the reaction to P_H 6.9, using 10% sodium carbonate, and boil for 30 minutes longer. Remove from the brine bath; make up loss from evaporation to 2 liters with distilled water. Filter through canton flannel and tube in 10 c c amounts. Autoclave at about 12 lbs. pressure for 10 minutes.

In preparing the plates, 5 c c of ascitic fluid, free from bile, and 0.5 c c of a 1 : 3,000 dilution of iodin-green (Grübler) are added to each tube of melted medium, just before pouring. The final reaction is generally about P_H 7.2. It should not be more alkaline than this.

If to be used in slants, the amount of agar is increased to 40 gm.

The following mediums contain a growth accessory principle. They are modifications of the "hormone" mediums, described by Huntoon,[4] and have been found very useful for the purposes specified. As beef heart constitutes the meat element, they may be prepared at slight expense.

B. Ascitic, salt-free, 1.5% peptone, "vitamine" agar. This medium has been employed in connection with the isolation of the gonococcus.

Five hundred gm. of fresh, chopped beef heart, free from fat (the beef heart meat should preferably be obtained directly from the slaughter house), one whole egg and 1 liter of distilled water are placed in a double boiler over a free flame and the temperature maintained at 60 C., with constant stirring, for 5 minutes. Fifteen gm. of peptone (Difco) and 18 gm. of flaked agar are now added and the temperature raised until the mixture assumes a brownish color. The medium is then made slightly alkaline to litmus, using a 10% solution of sodium carbonate. It is next placed in a flask, or preferably a coffee pot, and heated at 100 C. in the Arnold steam sterilizer for 1 hour. The clot is then separated from the sides of the receptacle, and it is replaced in the sterilizer for another hour. It may be cleared by centrifuging or by straining through a fine wire mesh and then through glass wool. A clear medium may often be obtained if the meat residue is deposited on the glass wool in a funnel and the fluid portion allowed to percolate through several times. As Huntoon has stipulated, neither cloth, cotton nor any other material with absorptive properties should be used in clarifying the medium. After filtration, the reaction is brought to P_H 6.8. It is then reheated and tubed in 10 c c amounts. It may be sterilized in the autoclave at 12 lbs. pressure for 10 minutes, but fractional sterilization at 100 C. flowing steam is preferable. In preparing the plates, 5 c c of ascitic fluid is added to each tube of melted medium, just before pouring.

A modification of this medium, prepared without ascitic or other serous fluid and which has been found useful in obtaining growths for complement-fixation tests, is described elsewhere.[1]

4 Jour. Infect. Dis., 1918, 23, p. 169.

C. A semi-solid agar with a growth accessory element (Huntoon). This medium, without the addition of ascitic fluid, has been found useful for maintaining our collection of stock strains. Even the most delicate strains of gonococcus have remained viable for 3 to 4 weeks without replanting, and the majority have lived for periods of 6 to 8 weeks or even longer, if kept at 37 C. The ingredients of this medium are the same as those stated in the formula of Huntoon.

Distilled water .... 1000 c c
Fresh chopped beef heart...................... 500 gm.
Peptone .... 10 gm.
NaCl .... 5 gm.
One whole egg

The same procedures are followed as in the preparation of medium B, and the final reaction is adjusted to P_H 6.8. This medium is tubed in about 7 cc amounts and is preferably sterilized in the Arnold sterilizer. The method of inoculating and the uses for this medium will be described. If ascitic fluid to the amount of about 1 cc per tube is added, the medium may be employed in connection with primary fishings of gonococcus colonies. It may also be used advantageously in the rejuvenation of delicate strains and in the recovery of old stock strains which have apparently died out.

CONDITIONS FAVORING MAXIMAL GROWTH OF THE GONOCOCCUS

Cole and Lloyd[5] in their analysis of the cultural requirements of the gonococcus, determined that there are three factors of prime importance: first, the concentration of the hydrogen ions, or the reaction; second, the concentration of amino acids; and third, the presence of certain growth stimulating hormones or vitamines. The following paragraphs contain a report of our experiments on the effect of these various factors on the growth of this organism.

Reaction.—Cole and Lloyd reported the mean optimal reaction for growth as P_H 7.6, with a possible range of hydrogen-ion concentration between 6.5 and 8.6. They also stated that with relatively simple (unfavorable) mediums the reaction is of great importance, whereas in mediums containing a growth stimulating substance, a wide reaction range is compatible with good growth. Our findings agree in general with these statements, although our point of optimal growth is nearer neutrality. This lack of conformity as regards the optimal point may be due in part to the use of different standards for determining the H-ion concentration. We have used the colorimeter methods of Clark and Lubs,[6] employing standard buffer solutions prepared according to their directions and which were, in turn, checked with solutions prepared in several other laboratories. They employed, on the other hand, the standards

[5] Jour. Path. & Bacteriol., 1916-17, 21. p. 269.
[6] Jour. Bacteriol., 1917, 2, p. 1.

of Cole and Onslow.[7] Warden,[8] several years ago, emphasized the importance of a slightly acid ($+0.7$ to 1.5 to phenolphthalein) reaction in mediums for the successful cultivation of the gonococcus. It is also our opinion that most investigators have employed mediums with a somewhat too alkaline reaction. Swartz[9] has recently reported successful growth on an ascitic agar medium with a H-ion range from 6.5 to 8, if this organism is cultivated under conditions of partial oxygen tension. Erickson and Albert,[10] using a beef testicular agar enriched with blood, found that the optimal reaction lay between P_H 7.4 and 7.6.

With our medium A, enriched with ascitic fluid, we have found that good growth may be obtained with a reaction ranging from about P_H 6.6 to 7.4, but if this medium is used without ascitic fluid the range is still more restricted toward the neutral point. With agar medium containing a growth accessory principle, a much greater latitude in reaction is permissible. The semisolid medium C has produced growth within a reaction range of from P_H 5.8 to 8.2, with an optimal zone between 6.4 and 7.7.

In table 1 are given both the degrees of growth at various H- ion concentrations with a stock strain of gonococcus, long under cultivation, and the remarkable period of viability observed in the lots of the medium with the more acid reaction. In this experiment 10 tubes of semisolid vitamine agar (C) of each reaction were seeded with gono-coccus strain 33; the tubes were sealed with paraffin and kept at 36-37 C. From time to time replants were made into this medium C, which had been enriched with ascitic fluid, or on ascitic agar slants. With so many of the original cultures to choose from in testing for viability, it was possible to plant either from a hitherto unopened tube or, at least, from an undisturbed part of the original growth in a tube which had been opened before. As table 1 indicates, a tube with an initial reaction of P_H 6.3 still held viable organisms after the lapse of a year. With this particular strain, too, successful growths were obtained for far longer periods from the tubes on the acid side of neutrality than from those on the alkaline. It should be noted, how-ever, that the initial reactions did not remain constant, but that the gonococcus growth caused a gradual change in the direction of alka-linity. On replanting from tubes which had been kept several months, the gonococcus colonies developed very slowly, no growth frequently appearing for 3 or 4 days. Cole and Lloyd[5] have also reported long

[7] Lancet, 1916, 2, p. 9.
[8] Jour. Infect. Dis., 1913, 12, p. 93.
[9] Jour. Urol., 1920, 4, p. 325.
[10] Jour. Infect. Dis., 1922, 30, p. 269.

viability periods for gonococcus when growing in stab culture in their 2% tryptamine blood agar with an H- ion concentration of about 7.6, some of their strains surviving from 67 days to 5½ months. Morax [11] found that ascitic agar stab cultures might retain their viability for 6 months. Warden,[7] using an artificial serum fluid medium, found that successful transplantation could be made after 100 days.

As the gonococcus surviving for one year without replanting was from an old stock strain and probably an unusually hardy one, a number of similar tests were conducted with recently isolated strains (table 2). As may be noted, the reaction of the medium, optimal for growth, was close to the neutral point. As regards the relation of reaction to viability, the tests with these strains yielded rather irregular results but, on the whole, at the periods of replanting a greater num-

TABLE 1

RELATION OF GROWTH TO VARIOUS REACTIONS AND THE RELATIVE PERIODS OF VIABILITY AT VARIOUS REACTIONS. UNENRICHED SEMISOLID MEDIUM C

H-Ion Concentration of Medium	Degrees of Growth	Viability Tests: Replants in Weeks After Primary Cultures									
		3	4	6	9	16	22	29	42	46	52
5.4	—										
5.8	+ +	+	+	+	+	+	+	+	+	+	—
6.3	+ + +	+	+	+	+	+	+	+	+	+	+
6.8	+ + +	+	+	+	+	+	+	—	—	—	—
7.4	+ + +	+	+	+	—	—	—	—	—	—	—
7.8	+ +	+	—	—	—	—	—	—	—	—	—
8.4	—										

ber of cocci were found viable in the tubes with a slight degree of acidity than in those with the more alkaline reaction. A difference in the character of the growth in relation to the reaction was also noted in connection with all the strains. After about a week's incubation, the growth on the slightly acid to neutral medium became thick, soft and slightly buff-colored, whereas on the alkaline side of neutrality it was thinner, white and generally drier in appearance. These differences were also noted by Cole and Lloyd. They observed, too, that autolysis apparently proceeds more rapidly on mediums more alkaline than that considered the mean optimum. Our observations add confirmation to this conclusion.

As to the relation of the reaction of the medium to viability, the tests with these strains yielded rather irregular results but, on the

[11] Ann. de l'Inst. Pasteur, 1918, 32, p. 471.

whole, at the times of replanting a greater number of cocci were found viable in the slightly acid tubes than in those with the more alkaline reaction.

These periods of viability (table 2), which are by no means the limits for even these delicate strains, contrast strongly with those obtained by others on slants of solid medium. Swartz [10] has reported a viability period of 7 to 10 days on slants of his ascitic agar medium and Cook and Stafford [12] of only 8 days with slants of their enriched.

TABLE 2

RELATION OF VARIOUS REACTIONS TO THE GROWTH OF RECENTLY ISOLATED GONOCOCCUS STRAINS AND RELATION OF REACTION TO VIABILITY *

H-Ion Concentration of Medium	Relative Degrees of Growth, Gonococcus Strains								Tests for Viability: Colony Development from One Loop					
	28	63	66	67	70	74	75	76	28 30 Days	66 21 Days	70 21 Days	74 45 Days	75 26 Days	76 26 Days
6.0	1	1	1	2	0	0	2	0	Numerous	Fairly numerous	—	—	∞	—
6.4	3	3	2	2	0	1	3	1	∞	0	—	Few	∞	∞
6.8	3	3	2	2	2	1	3	1	0	0	Very numerous	Numerous	Fairly numerous	∞
7.3	3	2	3	2	2	1	2	2	0	0	Very numerous	0	∞	Few
7.7	2	1	2	2	1	1	2	1	0	Fairly numerous	Numerous	Few	Numerous	Numerous
8.0	2	...	1	1	0	0	1	1	Fairly numerous	0	—	—	Fairly numerous	Fairly numerous
8.2	1	1	1	1	0	0	0	0	0	0	—	—	—	—
8.6	...	...	0	0	0	0	...	...	..	—	—	—	—	—

Strains 75, 76: third generation from isolation.
Strain 74: fourth generation from isolation.
Strains 63, 66, 70: fifth generation from isolation.
Strain 28: very delicate strain.
* Primary plants were made on semisolid agar (C) and tubes sealed with paraffin. Replants for viability were made on ascitic agar slants, using one uniform loop of the primary culture.

testicular agar, and no recoverable growth after 3 days with stab cultures. Hermanies,[13] however, using salt-free ascitic agar slants, about neutral to litmus, found that some of his strains would live for weeks or even months without transplanting.

Amino Acids.—Cole and Lloyd have emphasized the important influence of amino acids on the development of the gonococcus, especially in the presence of "growth hormones." Their amino acids were obtained by a tryptic digestion of casein and the product was desig-

[12] Jour. Infect. Dis., 1921, 29, p. 561.
[13] Ibid., 1921, 28, p. 133.

nated "tryptamine." In experiments bearing on this point we have used preparations from liver, casein and beef in which the amino acids constituted from 65 to 75% of the total nitrogen.[14] These products were used in 1% amounts in place of peptone in preparing the semi-solid medium C, and the growth and viability on these mediums were compared with that on the medium prepared with peptone, but otherwise identical. It was not found that these mediums, unusually rich in amino acids, produced a greater or a quicker growth; in fact, with some strains the results were inferior to those obtained with the peptone medium. Accordingly it was concluded that the peptone [15] used, especially if employed in a concentration of 1.5%, contained sufficient amino acids to effect the optimal development of the gonococcus. In fact, M'Leod and Wyon [16] recently reported that high concentrations of amino acids readily inhibit the growth of such organisms as pneumococci, meningococci and hemolytic streptococci.

Growth Stimulating Substances.—A third factor, considered by Cole and Lloyd of primary importance in the cultivation of the gonococcus, are two different growth stimulating substances, present in fresh blood and other materials, which were designated as "growth hormones." The substance of importance in inducing initial growth was considered a derivative of red blood cells and was shown to be readily absorbable by colloidal substances, such as agar and gelatin, and also by materials ordinarily used in clarifying mediums. The second substance, present in animal and plant tissues, was relatively non-absorbable, and was thought to stimulate luxuriant secondary growth. The first substance was considered to be of the same nature as vitamines or hormones because of the ease with which it was absorbed. As the term, vitamine, would seem to be a rather more appropriate designation for the substance stimulating primary growth, it will be employed hereafter.

Huntoon,[4] utilizing these principles of Cole and Lloyd, has described simpler methods for preparing these vitamine mediums. Instead of blood, beef heart or steak was employed with the idea that these tissues would provide sufficient of the growth accessory substances, especially when fortified with a whole egg. We have found mediums of this type useful in our work with the gonococcus, and have followed

[14] Prepared by the Arlington Chemical Co.
[15] Different lots of this peptone have varied somewhat in efficiency as growth producers, but only one sample was encountered which could not be used.
[16] Jour. Path. & Bacteriol., 1921, 24, p. 205.

Huntoon's methods with certain modifications in the adjustment of the reaction, the amount of peptone and the use of salt. Beef hearts have been used entirely as the meat base, and it has been deemed important to obtain them as fresh as possible. In all probability the primary growth stimulating substance is derived mostly from the blood content of the meat and in minor degree from the tissues.

The desirability of limiting the degree and duration of heating in the preparation of mediums of this type was emphasized by Huntoon on the supposition that the growth stimulating substances are to some degree heat labile. Thjotta and Avery,[17] in a careful analysis of the properties of the growth accessory substances essential for the cultivation of the hemophilic bacilli, determined that their V factor, derivable from blood and from yeast and other vegetable cells, was not impaired appreciably by boiling for 10 minutes, but their potency was greatly impaired by exposure in the autoclave to 120 C. for 30 minutes. In the preparation of these vitamine mediums for the gonococcus, it has been necessary to apply a boiling temperature for far longer periods than that specified by Thjotta and Avery as a safe limit. There can be no doubt, however, that the growth stimulating substances for the gonococcus survive this rather prolonged exposure to 100 C. in sufficient amount and degree to influence its development very favorably. That this is the case is shown by the experiments of Cole and Lloyd and also by our own results. The question arose, however, as to whether these growth accessory substances would resist the higher temperature of the autoclave. In order to test the effect of this heat factor a number of the most delicate strains in our collection were seeded on a particular lot of the semisolid vitamine agar (C) the several portions of which were subjected to the various degrees and durations of heating, as shown in table 3. Under column B of this table there is also demonstrated the adsorptive action of flannel and cotton on these accessory growth substances. Thjotta and Avery also found that their V factor, essential to the growth of B. influenzae, was readily adsorbed by bone charcoal.

The results reported in table 3 indicate that the growth stimulating substance in this medium is slightly impaired by the autoclave temperature of 120 C. for 5 minutes and seriously injured by a temperature of 120 C. for 30 minutes, and also by prolonged heating in the Arnold sterilizer. In estimating the degree of injury the results with lots A

[17] Jour. Exper. Med., 1921, 34, p. 97.

and B serve as good comparates; in the former the medium exhibits its maximal efficiency, whereas from the latter the growth stimulating element has been in large degree removed by filtration through absorbing substances.

Moisture.—It has long been realized that the gonococcus will not grow well on solid mediums from which the initial moisture has largely dried out. Only recently, however, has the importance of a moist air in the incubator been appreciated. Jenkins [18] has reported that a moist atmosphere, obtained by placing a large dish of water in the incubator, materially accelerates the growth of the gonococcus. Cook and

TABLE 3

THE EFFECT OF FILTRATION AND OF DIFFERENT DEGREES AND PERIODS OF HEATING ON THE GROWTH STIMULATING PRINCIPLE OF AGAR MEDIUM C *

Gonococcus Strains	A		B		C		D		E	
	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days
3	++	++	—	—	—	—	—	—	—	—
40	—	++	—	—	—	—	—	++	—	—
54	++++	++++	—	—	+	++	+++	++++	++	++
69	+	+	—	—	—	—	—	—	—	—
27	+++	+++	—	—	++	+++	+++	+++	+	++
41	++++	++++	++	++++	+	++	++	++++	++	++++
34	++++	++++	++++	++++	++++	++++	++++	++++	++	++++

+. ++, +++. ++++ indicate degrees of growth.
* Different portions of one lot of this semisolid medium treated as follows:
Lot A: Filtered through glass wool and heated at 100 C. in the Arnold Sterilizer for 20 minutes on 3 consecutive days.
Lot B: Filtered twice through flannel and absorbent cotton and sterilized as A.
Lot C: Filtered through glass wool and heated in the Arnold sterilizer for 2 hours on each of 3 consecutive days.
Lot D: Filtered as for C and heated in the autoclave at 120 C. for 5 minutes.
Lot E: Filtered as for C, and heated in the autoclave at 120 C. for 30 minutes.
The H-ion concentrations of these several lots after final sterilization ranged from PH 6.5 to 7.0 (within the zone of optimum growth).
Tubes of these several lots were seeded with one loop each of a saline suspension (about 3 billion strength) of gonococci. Strains 3, 40, 54, 69, 27 were of the delicate type.

Stafford [12] found that the best growths resulted when the culture tubes were placed in closed jars containing water and that placing a pan of water in the incubator was not sufficient. It has been our experience that a large bowl or pan of water (about 10 inches in diameter) pro‑ vides enough moisture for the ordinary sized incubator. There can be no doubt that the presence of such a moist atmosphere is a matter of prime importance, especially in obtaining primary growth from infected material. It acts as a marked accelerator of growth; colonies appear several hours earlier on the plates and in greater number than

[18] Jour. Bacteriol. & Path., 1921, 24, p. 160.

when incubated without special provision for moisture; in fact, there may be no growth at all in the presence of the ordinary dry air of the incubator.

As regards the physical state of solid medium, we have found that the best results are obtained when it is moderately moist and firm. For slants, after the proper amount of ascitic fluid has been added, the agar content should amount to about 1.5% and for plates rather less than that. Hall[19] has advised a hard, firm surface, free from excessive moisture, for optimal growth, but we believe that a very firm surface, such as is obtained with 2.5% agar, is decidedly unfavorable. Ascitic agar slants should be prepared the day before they are to be used and should generally be allowed to drain for an hour in the incubator before seeding. Culture plates should be poured several hours before they are to be streaked, or, if the medium has its full quota of moisture, may be prepared the day before.

Reduced Oxygen Tension.—Within recent years a number of investigators have advocated a reduced oxygen tension atmosphere as especially favorable for the growth of the gonococcus. This reduction in tension has been effected in various ways; through heating the air in the culture tube and closing tightly with a rubber stopper (Ruediger,[20] Swartz[9]); through a partial exhaustion of the air (Swartz[9]); through exposure to a CO_2 atmosphere (Chapin[21]); through the use of a bacterial culture with oxygen reducing properties, such as B. subtilis (Wherry and Oliver,[22] Herrold,[23] Hermanies[13]). From time to time we have tested these various procedures, but the results have been in no way superior to those obtained on the same medium exposed to moist air of normal pressure. The gonococcus in fact does not behave like an organism with a predilection for reduced oxygen tension. In stab cultures the growth in the stab is, at best, very feeble; in fact, in our semisolid medium it occurs exclusively on and immediately below the surface. It is worthy of note that all the procedures utilized for maintaining a reduced oxygen tension also tend to retain moisture in the medium or in the air, which is in itself an important factor in obtaining the maximal growth for the gonococcus.

[19] Jour. Bacteriol., 1916, 1, p. 343.
[20] Jour. Infect. Dis., 1919, 24, p. 376.
[21] Ibid., 1918, 23, p. 342.
[22] Ibid., 1916, 19, p. 288.
[23] Jour. Am. Med. Assn., 1921, 76, p. 225.

Cook and Stafford[11] and Erickson and Albert[9] have also recently reported that a reduced oxygen tension does not favor the growth of the gonococcus.

THE PRIMARY ISOLATION OF THE GONOCOCCUS

As it was seldom feasible to inoculate the culture plates immediately after the infected material had been obtained from gonorrheal cases, the following method was devised whereby a delay of several hours might elapse with a minimal risk of loss of viability on the part of the gonococci.

One c c portions of a mixture of 2 parts of semisolid "vitamine" agar (C) and 1 part of ascitic fluid are placed in narrow test tubes (6″ x ½″). This mixture is semifluid in consistency. After the swab has been infected with the gonorrheal discharge it is placed in a tube containing this medium, care being taken that the swab becomes well moistened with it, and is left there. This tube is then placed under the clothing; preferably next to the skin. It is advisable, although not essential, to warm the medium slightly just before introducing the swab. On reaching the laboratory the tubes containing the swabs are placed at once in the incubator. The plates, which should be slightly warmed, may be seeded then or the tubes may be left there for 3 or 4 hours before this is done. Within this period the gonococci apparently do not die out at all; in fact, they may begin to increase in numbers. The plating, however, should not be delayed so long that the contaminating bacteria have an opportunity to overgrow the gonococci. Even at room temperature gonococci in pus deposited on the surface of an ascitic semisolid agar tube and kept in a dimly lighted place have remained viable for a surprisingly long time. In 2 such experiments living gonococci were found after 48 hours in pus left under such conditions, although their numbers were reduced to a fraction of 1% of that originally present. After 3 days all had died. These specimens of pus were obtained from cases of acute urethritis in males and contained large numbers of gonococci. The tests offer a fair criterion of the probable maximal infectious period of such gonorrheal discharges, when kept moist at ordinary temperatures and removed from contact with strong light.

In taking specimens from cases of urethritis in males it was not found necessary to clean or treat the meatus in any way, especially if the discharge was taken up with a platinum loop. Pus from joint

cases should be distributed among 3 or 4 tubes of the semifluid ascitic agar, described in the foregoing paragraph, and plates seeded from them at once and after incubation for 24 to 48 hours.

In seeding plates the swabs are applied to only about one-fourth of the surface area of the medium. The swab should be rolled so that all parts come in contact with the medium. With a platinum loop the other three-fourths of the plate surface are now seeded by streaking from the part to which the swab was applied. In that way a proper distribution of colonies is likely to be obtained on some part of the plate. Before a second plate is seeded the swab should be reintroduced into the tube containing inoculated semifluid ascitic agar and so on for each plate.

In the preparation of plates for gonococcus isolation we have employed both A and B agar mediums, using with each 2 parts of the medium and 1 part of ascitic fluid. As is well known, some samples of ascitic fluid are unsuited for gonococcus culture. The lots used successfully were free from bile and had a specific gravity of 1.010 or higher. As will be explained presently, certain dyes have been used at times in conjunction with these mediums.

Colony Characteristics.—Medium A with ascitic fluid produces a colony which is rather different from that regarded as typical for the gonococcus. These colonies after 48 hours' incubation are only semi-translucent in texture and have a raised, even, or, at most, a slightly indented edge. They stand out prominently from the surface of the medium. The centers are somewhat thickened and exhibit few or numerous light colored granules. The edges are clear and homogeneous. By transmitted light they have a rather characteristic light fawn color with a suggestion of a greenish tinge. In consistency they are somewhat viscid or pasty. On primary isolation the colonies are generally just visible to the naked eye after 24 hours' incubation and are not characteristic in appearance. After 48 hours, however, they may attain a diameter of 1 to 3 mm. and are easily identified. They often continue to grow for about 10 days when they may reach a diameter of 8 mm. The older colonies have heaped up centers with thin spreading edges. One of the advantages of this medium is the unusual retention of viability even in the primary growth; successful replants have been made from such colonies up to 10 days. The gonococcus colonies are also distinctive in appearance and after a little experience are hardly to be mistaken.

The medium B with its growth accessory element may be used to advantage in conjunction with medium A. This plate medium may bring to development over 10 times as many gonococcus colonies as medium A. The colonies also develop more rapidly and may be visible in 18 hours. After 24 hours' incubation they frequently attain a diameter of about 1 mm. These colonies are colorless and translucent, showing a light smoky tinge by transmitted light. They may show heaped up centers or a flat surface. The edge may be very thin and slightly crenated or may be raised, well defined and smooth. The well isolated colonies exhibit the latter appearance and under low magnification show numerous light colored crumbs occupying the entire colony except the outer periphery. Whereas fishings from the A medium plates may be delayed for 3 or 4 days, the gonococcus-like colonies on the B plates should be fished by the second day as the rapid growth seems to be correlated with speedy disintegration. Among other organisms forming a colony similar to the one described in the foregoing, we have noted a gram-postive diplococcus and a small gram-negative coccoid bacillus.

Fishing of Colonies.—Fishings of colonies for the most part have been made into the ascitic semisolid medium (C). The growth on the surface of this medium is so characteristic as to be almost diagnostic in itself. The fished colony is seeded on and by short stabs all over the surface of this medium, which has the advantage of being not only favorable for the primary growth but also for the prolonged viability of the gonococcus. Of such original plants from the plates of 6 cases, the tubes of which had been sealed with paraffin, all were found alive after 50 days at 36 C. and 3 of them after 80 to 90 days. With this medium, then, there is no danger of losing a strain on first isolation and no need of making such frequent replants as has been considered necessary heretofore. It would seem entirely probable that this medium would prove as well adapted for fishing meningococcus colonies. After the first generation of the gonococcus on this ascitic semisolid medium, it is generally possible to transplant successfully to the medium without ascitic fluid; in a number of cases fishings have been made directly to the semisolid medium unenriched with ascitic fluid and growth obtained. Most strains, however, thrive only when the fishing is made to the enriched medium.

Selective Dyes.—Certain dyes, especially gentian violet and brilliant green, have proved so useful in the preparation of selective mediums for

various bacterial types that it was hoped some one might be found of service in the isolation of the gonococcus. A considerable number of dyes were tested and of these, iodin-green and methyl violet gave the more promising results. In most instances the test material consisted of swabbings from mild chronic vulvovaginitis cases in children. Frequently in such cases other types of bacteria are so numerous that isolation of pure cultures of gonococcus is a matter of great difficulty. In the experiments with iodin-green (Grübler), the dye was diluted 1 : 2,000 or 1 : 3,000 and 0.5 to 1 c c added to 15 c c of the ascitic agar medium A. It was observed that this dye, at the dilution used, exercised its inhibitory effect only when glycerol was present in the medium. Under these conditions the dye tends to suppress some types of gram-positive cocci—not streptococci—and certain diph-theroids. Colonies of staphylococci and diphtheroids which succeed in developing on the plates are tinted green, whereas the gonococcus colonies are either uncolored or show only a faint greenish tinge when viewed against a dark background. This dye, in the dilutions used, seemed actually to stimulate the growth of gonococcus as these colonies tend to become larger on plates containing the dye than on the control plates. In fact, the use of this dye in connection with this plating medium was finally adopted more on this account than because of its inhibitory effect on other bacteria, which, indeed, is rather slight.

Of the triphenylmethane dyes, gentian violet and methyl violet were the only ones tested extensively. Gentian violet suppresses effectively the great majority of gram-positive bacteria, but unfortunately gono-coccus, although a gram-negative organism, is also highly susceptible to its toxic action. The strongest dilution which could be used with any degree of success was a final one of 1 : 1,200,000 (1 c c of a 1 : 80,000 dye dilution in 15 c c of medium B). Although most gonococcus strains exhibit a slightly greater tolerance to the bacteriostatic action of gentian violet than do the majority of gram-positive cocci and diphtheroids, the margin of difference is so slight that the use of this dye as a selec-tive agent offers slight promise of success. Occasionally, when using gentian violet medium in making cultures from gonorrheal discharge, we have obtained a plate showing a nearly pure culture of gonococcus with a marked suppression of the gram-positive organisms in the speci-men, but comparison with the control plate would indicate that only a very small percentage of the viable gonococci in the pus had developed

into colonies. On the gentian violet plates, also, cultures did not become visible to the naked eye until the second or third day of incubation. Cook and Stafford[12] have recently reported on the use of gentian violet and also other triphenylmethane dyes as ingredients of a selective medium for the gonococcus. Gentian violet was used in connection with testicular or chocolate blood testicular agar in a dilution of 1 : 500,000. The results obtained, however, in the application of this medium to clinical diagnosis were not promising.

As is well known, Churchman[24] has advocated the use of gentian violet in the local treatment of cases of purulent arthritis including the gonorrheal type. In discussing the results obtained, he observed that as the gonococcus is a gram-negative organism it might be expected to be relatively unaffected by gentian violet, but he was unable to settle this point experimentally. Our experiments would seem to indicate, however, that the gonococcus exhibits only a slightly lower degree of susceptibility to the bacteriostatic action of gentian violet than do the gram-positive cocci and hence should be classified as a "gentian violet positive organism."

In connection with the isolation of the gonococcus, methyl violet has yielded somewhat better results in our hands than has gentian violet. In our more successful experiments this dye has been diluted to about 1 : 125,000 and 1 c c added to 15 c c of the ascitic agar medium B. A stronger dilution than this tends to be too inhibitory of the development of gonococci. Staphylococci have been quite effectively suppressed as have also some troublesome types of diphtheroids and spore-bearing bacteria. On the other hand, streptococci and gram-negative bacilli are not affected at all. This dye, accordingly, is only to a limited degree selective for the gonococcus, but we have found at times that its presence in the medium has permitted the isolation of this organism when the control plates were covered with growth of other bacterial types. The advantages and limitations in the use of this methyl violet medium may be estimated from the results reported in a following article.[25] As will be observed, it may be used to some advantage in connection with other plating mediums but dependence should not be placed on it alone. This dye has been used only with the ascitic "vitamine" agar B. The optimal dilution should be determined for each

[24] Ibid., 1920, 75, p. 583.
[25] Jour. Infect. Dis., 1922, 31, p. 148.

sample of dye used. Erickson and Albert [9] have recently reported that of various violet and green dyes tested, methyl violet was the most effective for isolation of the gonococcus.

Reaction.—The final reaction of these plating mediums has always been close to P_H 7.2.

CRITERIA FOR THE IDENTIFICATION OF THE GONOCOCCUS

If the material for culture has been obtained from the genito-urinary tract, we have found that the type of colony formation together with morphology and staining reaction is an almost infallible guide to the identification of the gonococcus. As has been mentioned, only one or two types of bacteria from this locality form colonies closely resembling that of the gonococcus and in no case do these bacteria bear a resemblance morphologically to this organism. Cultures from several hundred cases of gonorrhea have never shown any colonies of M. catarrhalis or any other diplococcus morphologically resembling the gonococcus, with its typical picture of a mixture of well-staining, biscuit-form diplococci and swollen, more or less completely autolyzed, irregularly staining cocci and diplococci. Although this is the case, we have not depended on these points alone for identification.

Inability to grow on unenriched medium during the first few generations has been generally accepted as an important diagnostic test. The great majority of gonococcus strains will conform to this requirement, but occasionally we have encountered undoubted gonococcus strains which would grow slowly on ordinary glycerol, beef infusion, peptone agar slants with a reaction of P_H 7.4, on planting from the second or third generation of subcultures. These strains were .mostly isolated from vulvovaginitis infections in children. Some eyars ago Wollstein [26] reported that strains from such sources grew readily on plain agar. This capacity, however, may not be considered as a cultural feature differentiating strains causing infantile vulvovaginitis from those concerned in the gonorrhea of adults, for we have isolated from these children's cases strains of a type which was very delicate and difficult to cultivate. No gonococcus strain, of course, will grow at room temperature on even the most favorable type of medium. Serologic tests, such as agglutination and agglutinin absorptions, cannot be depended on as a certain guide to identification, as has been explained elsewhere. That fermentation tests are of great value in the differentiation of the

[26] Jour. Exper. Med., 1907, 9, p. 588.

gram-negative diplococci has been demonstrated by Elser and Huntoon and others. As will be shown presently, our tests with over 80 strains proves that the gonococcus is capable of splitting glucose alone and is thus to be differentiated from its nearest relative, the maltose fermenting meningococcus.

The characteristics distinguishing the gonococcus from other similar organisms may then be summarized as follows: appearance of the colony, reaction to the gram stain and morphology, inability to grow at room temperature and typical fermentation reactions.

FERMENTATION TESTS

Elser and Huntoon,[27] in an extensive series of fermentation tests with different species of gram-negative diplococci capable of a parasitic existence within the human body, found that of the 10 carbohydrates employed in the tests, the gonococcus was the only representative of this group which fermented glucose. In this conclusion they confirmed the earlier finding of Rohe [28] that of glucose, maltose and levulose, the gonococcus splits glucose alone. Within recent years there has been a general agreement that the gonococcus does not ferment maltose and by that fact may be differentiated from the meningococcus. As to the action of gonococcus on galactose, however, there is no such uniformity of opinion. The earlier observers, Dunn and Gordon,[29] Arkwright [30] and Sherman and Ritchie,[31] all report the fermentation of galactose by gonococcus. Elser and Huntoon, however, obtained negative results in tests with 15 strains of this organism. More recently Cole and Lloyd [5] reported that galactose was fermented by the gonococcus but that the acidity was not as great as when glucose was the test sugar. As a probable explanation of the discrepancies in these findings certain experiments of Elser and Huntoon with galactose may well be cited. They found that with intermittent streaming steam sterilization for the customary time periods, galactose and also levulose in a menstruum containing very small amounts of free alkali were likely to be hydrolyzed with the production of acidity, and that this change continued in some instances when the medium was exposed to incubator temperature. However, by using glassware devoid of free alkali and by sterilizing the

[27] Jour. Med. Research, 1909, 20, p. 377.
[28] Centralbl. f. Bakteriol., O., 1908, 46, p. 645.
[29] Brit. Med. Jour., 1905, 2, p. 421.
[30] Jour. Hyg., 1907, 7, p. 145.
[31] Jour. Path. & Bacteriol., 1908, 12, p. 456.

,sugars separately through exposure of the distilled water solutions to live steam for 10 minutes, they demonstrated that these hydrolytic changes may be avoided.

As a base for our fermentation tests we have employed a sugar-free broth plus ascitic fluid. Beef infusion was made sugar-free by planting with B. coli and incubating for 24 hours. To the filtrate was added 1% peptone and 0.5% NaCl. After adjusting the reaction to P_H 7.0, the medium was tubed in 5 c c amounts and autoclaved. One c c of ascitic fluid was then added to each tube. The ascitic fluid used had been in cold storage for over 4 months, a period sufficiently long to permit the complete disappearance through hydrolysis of any fermentable carbohydrate which it might have contained. 12% solution of the sugar in distilled water was exposed to flowing steam at 100 C. for 12 minutes and 0.5 c c was added to each tube of the ascitic broth. These tubes of medium were then incubated for 3 days at 37 C. as a test for sterility. The consistent results obtained with levulose, galactose and maltose indicate that these relatively unstable sugars were not injured by the degree and period of heating applied in sterilization.

In these comparative fermentation tests we have used a fluid medium because it permitted a determination of the exact degree of change in reaction through the use of colorimetric methods. As a routine method, however, a solid or semisolid medium to which an indicator has been added is to be preferred as the growth of the gonococcus is much more rapid on such a medium and a reading may be made within 24 to 48 hours. A medium of this type, which has given satisfactory results, is described in a following paragraph. As is indicated in table 4, reaction readings for these gonococcus strains in the various fluid sugar mediums were made after 7 days' incubation. The H-ion concentrations were determined through the use of bromthymol-blue or phenol red and comparisons with their respective scales as the conditions called for.

In this tabulation of fermentations (table 4) the results with 60 gonococcus strains are given in detail. In addition 25 gonococcus strains isolated from cases of mild chronic gonorrhea in women were tested on glucose and maltose, making a total of 85 strains. These strains had been isolated from a great variety of clinical conditions, including acute and chronic urethritis in males, vulvovaginitis in children, arthritis cases, septicemias and cervix uteri infections. They also represented widely separated geographical localities: many parts of this country and such foreign countries as Mexico, England, France, Bel-

TABLE 4

FERMENTATION TESTS WITH 60 GONOCOCCUS STRAINS [*]

Gonococcus Strains	Glucose P$_H$	Galactose P$_H$	Maltose P$_H$	Levulose P$_H$
1	6.3	7.1	7.3	7.4
2	6.6	7.2	7.3	7.4
3	6.4	7.0	7.1	7.5
4	6.0	7.1	7.3	7.4
5	6.3	7.2	7.5	7.6
6	6.4	7.2	7.3	7.3
7	6.2	7.2	7.8	7.8
8	6.5	7.2	7.3	7.4
10	6.4	7.0	7.3	
11	6.2	7.2	7.3	7.4
12	6.2	7.4	7.5	7.3
13	6.0	7.0	7.2	
14	6.4	7.5	7.5	7.8
15	6.2	7.4	7.5	7.4
16	6.5	7.0	7.2	7.1
17	6.2	7.5	7.5	7.6
18	6.2	7.8	7.6	7.3
19	6.7	7.5	7.5	8.2
20	6.2	7.0	7.2	7.3
21	6.2	7.1	8.0	7.6
22	6.6	7.1	7.3	7.3
23	6.4	7.6	8.0	7.3
24	6.5	7.1	7.2	7.3
25	6.1	7.2	7.5	7.4
26	6.0	7.3	7.2	
27	6.3	7.1	7.4	7.3
28	6.2	7.1	7.6	7.3
29	6.2	7.5	7.3	7.8
30	6.0	7.1	8.0	
31	6.2	7.8	8.1	
32	6.0	7.2	7.2	
33	6.0	7.1	7.2	
34	6.0	7.6	7.2	
35	6.7	7.4	8.0	
36	7.2	8.0	7.4	
37	6.2	7.2	8.0	
38	6.2	7.2	7.2	
39	6.2	7.3	7.3	
40	6.5	6.9	7.1	
41	6.2	7.2	7.4	
42	6.2	7.2	8.2	
43	6.3	7.1	7.4	
44	6.2	7.3	7.8	
45	6.3	7.3	7.3	
46	6.5	7.2	7.8	
47	6.5	7.2	7.3	
48	6.2	...	7.8	
49	6.2	7.3	7.3	
50	6.2	7.1	7.4	
51	6.4	7.2	8.0	
52	6.0	7.3	7.2	
53	6.2	7.1	7.2	
54	6.2	7.3	7.4	
55	6.2	7.4	7.8	
56	6.5	7.4	7.9	
57	6.3	7.2	7.2	
58	6.5	7.4	7.8	
59	6.4	7.1	7.3	
60	6.7	7.4	7.1	
61	6.2	7.4	7.8	
Control: meningococcus	5.9	7.3	5.8	
Control 1 (sugar mediums uninoculated and incubated)	7.2	7.1	7.2	7.4
Control 2 (medium without sugar inoculated with gonococcus and incubated)	7.2			

[*] All tests for H-ion concentrations were made after 7 days' incubation.

gium, Germany and Egypt. As may be noted, with one exception these strains all produced a definite acidity in the glucose broth medium. The strain failing to split this sugar was an old stock culture (strain "G," redesignated 36). This strain grew very well in the medium but in repeated tests failed to attack the sugar at all. It would seem likely that we have here an instance of repressed or lost function rather than an exception to the general rule that the gonococcus ferments glucose. The strains differed considerably in the amount of acid produced but this variation was not definitely correlated with either the age of the strain or the amount of growth; some of the strains producing only a slight amount of acid were among the most vigorous growers.

As is indicated in table 4, none of the strains tested fermented galactose, maltose or levulose. The results with galactose are of rather more academic than practical interest as, according to the results of Elser and Huntoon, none of the gram-negative diplococci, which bear a close resemblance to the gonococcus, split this sugar. On the other hand, the uniform absence of action on maltose adds confirmation to the value of this sugar for the differentiation of the gonococcus from the meningococcus. The meningococcus strain, used as a control, acted promptly on this maltose medium, producing a marked degree of acidity in 48 hours. No recent observer has definitely claimed that the gonococcus may ferment maltose. Hermanies [12] recently reported in reference to a large number of gonococcus strains that "practically none of them fermented maltose," the exceptions apparently being 2 strains giving rise to a slight initial acidity which gave way after 48 hours to alkalinity. Wollstein,[26] in 1907, claimed that 10 strains of gonococcus from cases of vulvovaginitis in infants, all fermented maltose. Our tests, however, have indicated that such infantile strains do not differ in their fermentations from strains isolated from adults; 8 such strains (48, 54, 55, 56, 57, 58, 60, 61) gave positive results with glucose and negative results with galactose and maltose.

In the galactose and levulose medium, and to a rather more marked extent in the maltose medium, some degree of alkalinity was produced by the majority of the strains after 7 days' incubation. This degree of alkalinity was quite definitely linked with the vigor of the growth.

Our results have shown definitely that the presence of a sugar fermentable by the gonococcus in a medium does not enhance its growth, for fully as vigorous growth occurred in this fluid medium without any sugar or with the nonfermentable sugars as in the glucose tubes. The

same finding also held true for solid mediums. We can thus confirm the conclusion of Cole and Lloyd [5] that the addition of glucose to mediums prepared for gonococcus culture is not desirable.

A few of our gonococcus strains grew so poorly in this fluid medium that it could not be used for determining their fermentative activities. This medium is also not well adapted for diagnostic tests as the necessary incubation period covers several days. We have found that a sure growth with a definite reading after about 24 hours' incubation may be obtained by using a semisolid agar medium to which brom-thymol-blue has been added as an indicator. This medium is prepared with meat-infusion, sugar-free, 1.5% peptone broth to which 2/3% agar is added. To this semisolid agar, adjusted to P_H 7.0, is added brom-thymol-blue in an amount sufficient to give a fairly deep color. It is tubed in 5 c c amounts, sterilized, and the ascitic fluid and sugars added as advised for the fluid medium. The gonococcus growing on the surface of this unslanted medium causes, within 24 to 48 hours in the presence of glucose, a definite change in color from bluish green to yellow in the medium immediately below the growth. With some strains the change to yellow is evident within 18 hours. In the presence of maltose or other nonfermentable sugar the color either remains unchanged or a bluish tinge develops. This dye indicator does not inhibit the growth of the most delicate strains.

MAINTENANCE OF STOCK STRAINS OF GONOCOCCUS

The maintenance of a large collection of gonococcus strains on ascitic agar or blood agar slants is a troublesome matter and necessitates their being transplanted at intervals of a week or less, in fact some investigators [9] have advised daily replanting of stock cultures. We have found that these difficulties may be in large measure eliminated by the use of the semisolid medium C, which contains a growth accessory principle and is prepared, with slight modifications, according to the method of Huntoon. In using this medium the gonococcus growth is seeded into the upper one-fourth inch or so of the unslanted agar, which has a H-ion concentration of about 6.8. One of the great advantages of this medium is that it is sterilizable, and one may thus avoid the contaminations which are so prone to occur if ascitic fluid or other nonsterilizable albuminous fluids are used; also this medium does not dry out, and hence favors the development of a thick moist growth with most gonococcus strains. With stab cultures into this medium, the

growth is limited to about 4 mm. below the surface, none occurring in the depths of the stab. Huntoon also recommended a semisolid medium of this type for the preservation of stock cultures, reporting a viability period of 3 months for the meningococcus and 2 months for the gonococcus.

The viability factor with this medium has already been discussed from the standpoint of various H-ion concentrations. As was pointed out in that connection, some strains remain viable in this medium for periods ranging from several months to one year; other strains of the more delicate type may not give successful replants in this unenriched medium longer than one month. As a matter of routine, accordingly, the stock strains of the gonococcus have been replanted about every three weeks. Any strain failing to grow could always be recovered by the use of this medium enriched with ascitic fluid (see p. 126). These exceptionally delicate strains exhibited on first isolation the common characteristics of producing an unusually viscid growth with rapid autolysis of the cocci. On prolonged cultivation, however, most of them tended to lose these characteristics and to become much more hardy.

Young growths of gonococcus on this medium placed at room or icebox temperature tended to die out quickly. The limit of viability at both these temperatures ranged from less than 3 days to not more than 6 days. Hermanies, on the other hand, using ascitic agar slants, found his stock strains of gonococcus uniformly viable after 8 to 10 days at room temperature, and some remained so for periods up to several months. Cook and Stafford, using an unenriched testicular agar, obtained a viability period limited to 8 days, regardless of whether the cultures were kept at incubator, room or icebox temperatures. We have found the optimal temperature to be between 36 and 37.5 C., although continued viability is possible with temperatures up to 38.5 C.

SUMMARY

For the optimal growth of the gonococcus the reaction of the medium should be set close to the point of absolute neutrality; between P_H 6.8 and 7.4. The reaction range, however, compatible with growth on a semisolid medium containing a growth accessory factor was found to extend from P_H 5.8 to 8.2.

The relation of viability to the reaction of a medium was studied. A slightly acid reaction was found, on the whole, more favorable than

a slightly alkaline reaction. The remarkable retention of viability for one year was noted in reference to one strain seeded on a semisolid "hormone" agar (Huntoon) with a primary reaction of P_H 6.3.

No better growth was obtained by the use of a medium containing a high concentration of amino acids than when prepared with the specified amount of peptone.

The presence of glucose does not enhance the growth of the gonococcus.

The growth stimulating principle in a medium prepared according to Huntoon's method was found to be slightly impaired by exposure in the autoclave to 120 C. for 5 minutes and seriously injured, but not entirely destroyed, after 30 minutes at that temperature.

Abundant moisture in the air of the incubator is a prime requisite for the optimal growth of the gonococcus, especially on first isolation, but a reduced oxygen tension was not found to be advantageous.

Fermentation tests constitute the most valuable single criterion for the differentiation of gonococci from other similar gram-negative diplococci. No one of 86 gonococcus strains tested split maltose, and all but one fermented glucose. None of the strains tested on levulose and galactose split these sugars. A sugarfree, semisolid, ascitic agar medium, with bromthymol-blue as an indicator, has proved satisfactory as a base for fermentation tests. Differential readings may be made after 18 to 24 hours' incubation.

A semisolid, sterilizable medium with a growth accessory factor (Huntoon formula) was found admirably adapted for carrying a large collection of gonococcus strains. Replantings have not been necessary oftener than once in three or four weeks.

Two plating mediums are described which have been found serviceable in the isolation of the gonococcus. One of these mediums is made in some degree selective by the incorporation of a dye, iodin-green. For the best results the reaction is of prime importance; the final H-ion concentration should be about 7.2. A method of collection of gonorrheal pus specimens for culture is described.

COMPARATIVE VALUE, FROM STANDPOINT OF PUBLIC HEALTH, OF SMEARS, CULTURES AND COMPLEMENT FIXATION IN THE DIAGNOSIS OF CHRONIC GONORRHEA IN WOMEN *

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In the first part of this article are reported the results which have been obtained in the application of the cultural methods of Torrey and Buckell [1] to the diagnosis of cases of chronic gonorrhea in women, and in the second part, a comparative study of the relative values, from public health standpoint, of smears, cultures and complement-fixation tests in the diagnosis and control of such cases is presented. The patients were prostitutes who had received a court sentence.* *

1. *Cultural Results.*—As it was not feasible to seed the culture plates immediately after obtaining specimens from these cases, it was necessary to devise a procedure through which the viability of any gonococci present in the discharges, on the swabs, would be preserved during the period occupied in transporting them to the laboratory. A method which proved satisfactory is described in the article referred to. The plates should be poured at least 2 or 3 hours before they are to be streaked. After seeding, they are placed in an incubator in which the air is kept very moist and the temperature at 36 to 37 C. It is seldom worth while to attempt fishings from the plates after only 24 hours' incubation as the gonococcus colonies are frequently very small—barely visible to the naked eye—and not particularly characteristic. After 48 hours, however, the colonies have generally assumed a typical appear-

ance and those resembling the gonococcus type should be fished. It is best not to delay the fishing from medium B plates beyond the second day as the gonococci tend to die out rather quickly on this medium. In some instances the gonococcus colonies have grown up very slowly, and accordingly all the plates should be incubated and inspected daily for at least 5 days. The isolation of the gonococcus in pure culture in some cases was a matter of considerable difficulty owing to the presence of large numbers of other colonies on the plates. Pure strains were always obtained, however, by replatings on one or the other of the dye-containing mediums. The appearance of the gonococcus colonies on these mediums, the method of fishing the colonies and the criteria for the identification of the gonococcus have been discussed in the preceding paper.

For each case 4 plates were generally used, prepared as follows:

1. Medium B, 10 c c plus 5 c c ascitic fluid plus 1 c c methyl violet, 1 : 100,000 dilution in distilled water.

2. Medium B, 10 c c plus 5 c c ascitic fluid.

3. Medium A, 10 c c plus 5 c c ascitic fluid plus 0.5 c c iodin-green, 1 : 3,000 dilution in distilled water.

4. Medium A, 10 c c plus ascitic fluid, 5 c c.

As is indicated in table 1, rather more successful isolations were effected with plates 3 than with any of the other combinations, although plates 2 yielded nearly as good results and in 4 instances showed gonococcus colonies when the other plates were negative. These 2 plates, however, were more frequently overgrown with contaminating bacteria.[2] In only 2 instances was a successful isolation obtained from plate 1 when the other 3 proved negative, and in many instances no gonococcus colonies developed on plate 1 when one or more of the other plates were positive. Streptococci gave a good deal of trouble and were present in much greater numbers in the specimens from the cervix uteri than in those from vulvovaginitis in children or from cases of urethritis in males. The methyl violet, in the strength employed, frequently did not inhibit the development of the streptococci nor certain prevalent types of diphtheroids, but too often did inhibit the growth of gonococci. It would seem, perhaps, hardly worth while to employ plates 1 in routine work on women. Plates 4, also, proved less effective than did the same medium with the dye, iodin-green. In view of these

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[2] An effective method for limiting the overgrowth of plates with spreading types of bacteria (B. subtilis, etc.) consisted in ringing such colonies with gentian violet stain, applying the stain with a swab around the colony.

results we would recommend the use of mediums 2 and 3 in connection with gonorrheal cases of this type, employing at least one plate of the former and two plates of the latter for each specimen.

Of the total 102 women from whom cultures were made, successful isolations were effected in 29, or 28.4%.

TABLE 1

RESULTS OBTAINED WITH THE FOUR VARIETIES OF MEDIUMS EMPLOYED IN THE ISOLATION OF GONOCOCCUS

Case	Plate 1 Ascitic Agar B + Methyl Violet	Plate 2 Ascitic Agar B	Plate 3 Ascitic Agar A + Iodin-Green	Plate 4 Ascitic Agar A
L. Sm.	−	+	−	−
L. S.	−	+	−	−
P. A.	−	−	−	+
E. B.	−	−	+	−
N. C.	−	−	+	+
M. D.	−	+	+	−
L. R.	−	+	+	+
L. D. cerv.	+	+	+	−
ureth.	+	+	−	−
S. B. cerv.	−	−	+	−
ureth.	+	−	−	−
A. R.	−	C	+	C
A. M.	−	+	+	+
G. S.	−	−	+	+
A. Mac.	+	+	+	+
F. H.	+	+	+	+
C. J.	−	−	+	−
J. F.	−	C	+	−
M. Do.	+	+	+	+
H. M.	−	+	+	−
R. G.	−	C	+	C
R. C.	−	+	−	−
A. D.	−	+	+	−
M. H.	−	−	+	−
M. W.	+	+	+	−
J. V.	..	..	..	..
V. M.	−	+	+	−
R. Ca.	C	C	C+	C
G. W.	−	+	−	−
V. G.	−	+	+	−
L. S.	−	−	+	−

+ indicates gonococcus colonies on the plate; C indicates plate badly contaminated.

The strains isolated from our 29 positive cases were definitely identified as gonococci. In 5 instances pure cultures were obtained and identified on the basis of type of colony growth, morphology, and reaction to gram stain, rapid autolysis and short period of viability, but the strains were lost before further tests could be applied. The other 24 strains were all subjected to fermentation tests and a considerable number of them to agglutination tests with a polyvalent gonococcic serum. We believe there can be no question in regard to the identity of them all.

From the standpoint of control of venereal infection it is of interest to find that 9 of the 29 patients with positive cases harbored the gonococcus in the cervix uteri without any definite symptoms of gonorrhea. Also, although in most of these positive cases the number of viable gonococci was very small, there was no definite correlation between the number of gonococcus colonies on the plates and the clinical picture; that is to say, a few of the clinically doubtful cases gave fairly numerous gonococcus colonies on the plates, whereas certain other cases showing quite definite clinical symptoms yielded exceedingly few gonococcus colonies and those only on one plate. We have made no comparative virulence tests on the gonococcus strains isolated. It is possible that those from the clinically doubtful cases would prove to be relatively avirulent. Jötten[3] has recently reported that strains from cases of gonorrhea with complications are, as a rule, much more virulent, as indicated by the inoculation of white mice, than those cases with milder symptoms.

In regard to 92 of the patients there are official records of the results of gram-stained smears. Accordingly, we may present at this point a comparative statement bearing on the relative values of these two methods of diagnosis. In most instances smears from the urethra and the cervix uteri were prepared at the time the specimen for culture was obtained. In addition, from most of the cases smears were taken at other times. In the tabulation the report most suggestive of gonorrhoeal infection has been selected. In diagnosing these smears the rules of Williams and Wilson[4] were followed: positive smears show leukocytes filled with morphologically typical gonococci; suspicious smears show some intracellular organisms suspiciously like gonococci and 50% or more of polymorphonuclear leukocytes; observation smears show 50% or more of polymorphonuclear leukocytes, but no suspicious intracellular diplococci.

Of these 92 different cases, 26, or approximately 28%, yielded positive cultures. Among these cases there was one acute infection and 8 subacute. The majority, or 59, presented the clinical picture of chronic gonorrhea, as described by Smith and Wilson,[5] with at least 21 cases for which a diagnosis of "doubtful gonorrhea" was given. Gram-stained smears from these patients yielded a positive diagnosis in 13

[3] München. med. Wchnschr., 1920, 67, p. 1067.
[4] Collected Studies from Research Laboratory, Dept. of Health, City of New York, 1911, 6, p. 29.
[5] Jour. Immunol., 1920, 5, p. 499.

instances, or in just one-half as many as did the cultural method. Of the 26 culturally positive cases, 5 gave positive films, 2 were suspicious, and 19 were reported as of the observation type. There were, accordingly, 8 cases yielding positive smears from whom the gonococcus was not isolated.

Twenty-six of the patients had received irrigations for longer or shorter periods before the cultures were made, although, of course, the local treatments were stopped 3 or 4 days before the specimens were taken. Of these treated patients only 3 yielded positive cultures, but 5 gave positive smears. Further, the 5 cases giving positive smears were not among those successfully cultured; of the latter, 2 gave the observation type of smear and 1 the suspicious type.

It should be noted that in some of these cases the plates were so overgrown with other types of bacteria that gonococci, even if present, would not have had an opportunity to develop. If a second examination had been made in some of these cases no doubt a somewhat higher percentage of positive results might have been obtained. On the other hand, the smears were not always of such a quality as to provide optimal conditions for examination.

These results are summarized in table 2.

TABLE 2

COMPARATIVE RESULTS WITH CULTURAL AND SMEAR METHODS OF EXAMINATION

Total Number of Cases	Cultural Results		Gram-Stained Smears			
	Positive	Negative	Positive	Suspicious	Observation Cases	Negative
92	26	66	13	17	56	6

From our results with these two methods of diagnosis in suspected gonorrheal infections of this type we may conclude that the cultural methods employed are likely to give a higher percentage of definitely positive findings than are the smear examinations, but on the other hand, the latter may be positive when cultures are negative. Of the 21 cases clinically diagnosed as "doubtful gonorrhea," 5 gave positive cultures and 3 positive smears and 4 suspicious smears, but 4 out of the 5 culturally positive cases yielded the observation type or negative smears. These two methods, thus, tend to supplement each other and are both of value in the control of these cases.

2. *Comparison of the Complement-Fixation Test with Smear and Culture in Relation to Clinical Diagnosis.*—In this section are reported the results obtained with complement-fixation tests by Miss Wilson of the Serologic Laboratory, Department of Health, the smear examinations at the Kingston Avenue Hospital, and the cultures by Mr. Buckell, on 56 of the 102 cases described in part 1.

The method for the complement-fixation test has been described.[5] The essential points are the preliminary tests of individual guinea-pig serums for gonococcus fixability and careful titration of the selected, pooled complement with constant doses of antisheep amboceptor and 5% suspension of sheep cells.

TABLE 3

COMPARISON OF SMEAR, CULTURE AND COMPLEMENT FIXATION IN VARIOUS STAGES OF GONORRHEA IN WOMEN

Diagnosis	Number of Cases	Percentage of Positive Diagnoses		
		Smears	Cultures	Complement Fixation
Acute gonorrhea......................	1	Observation	100% positive	No complete fixation
Subacute gonorrhea...................	8	50%	50%	. 50%
Chronic gonorrhea....................	33	12%	20%	69.5%
Doubtful gonorrhea..................	14	14%	28%	71%

A summary of the tests of the 56 cases without reference to the clinical data, shows 16 cases giving positive cultures, or 27%, 10 giving positive smears, or 16.5%, and 37 giving fixation, or 66%. The fixations were divided into: 8 strongly positive, or 14%; 15 moderately positive, or 26.5%, and 14 weakly positive, or 25%. If we should consider the weakly positive reactions insignificant, we would still have 40.5% positive fixations in the 56 cases. In the discussion of the following tables we shall give our reasons for considering the weakly positive reactions to be diagnostic of present or recent gonorrheal infection.

The important point is to determine the relation of the laboratory tests to the clinical classification, and we give a comparison in table 3. The clinical classification is that used at the Kingston Avenue Hospital as described by Smith and Wilson.[5] This classification cannot be taken as a hard and fast guide to the course of the disease because of the inability to obtain correct histories and dates of infection of the patients.

As was demonstrated by Schwartz and McNeil,[6] in the acute and subacute stages of gonorrhea one would not expect to obtain as high a percentage of positive reactions as in the chronic stage.

It is of interest to compare our results with those of Smith and Wilson, who carried out a similar comparative investigation, using for the cultural work a glycerol-veal-horse-serum agar streaked with blood. Of a total of 50 cases, 7 were positive culturally, 14%; 3 were positive by smear and 26 had smears of the observation type; 41 gave positive fixation, or 82%, with 8, or 16%, showing a strong positive reaction.

Wilson has made a further study (to be published) of 181 cases previous to the present series. The 181 cases were classified as:

54 subacute cases[7] having 46.5% positive smears,
49.5% positive cultures,
51.5% positive fixation.
85 chronic cases having 13% positive smears,
15% positive cultures,
76% positive fixation.
7 doubtful clinically no positive smears,
no positive cultures,
42% positive fixation.

Controls: 346 nongonorrheal patients gave no trace of fixation, and 4 normal laboratory workers gave no trace of fixation.

In these three studies the complement fixation reactions in chronic cases dropped from 82% in first study to 76% in second study and to 69.5% in the present study.

We have not been able to determine the exact reason for this decrease in the positive reactions, but the chief cause may have been due to the time in the course of the disease that the tests were made. The character of the patients in our service has changed somewhat during the past two years. At the time of our first study all prostitutes having venereal disease were sent to Kingston Avenue Hospital, while, for some time past, the first offenders and young girls, only, are sent there.

In table 4 is given a report of the laboratory tests on 16 of our culturally positive cases. In these the complement-fixation tests were made at the serologic laboratory. The remainder of our culturally positive cases had the complement-fixation tests performed at another laboratory and are not included in this table.

[6] Am. Jour. Med. Sc., 1912, 144, p. 815.
[7] Included in the 54 subacute cases are 11 diagnosed as acute on admission, but as these were not early acute they have been placed with the subacute cases.

TABLE 4

RESULTS OF COMPLEMENT FIXATION AND SMEAR EXAMINATIONS IN 16 CASES
CULTURALLY POSITIVE

Case	Complement Fixation			Smears	
	Positive	Negative	Doubtful	Urethra	Cervix
L. S.	+	..	..	Suspicious	Suspicious
L. Sen.	+	..	..	+	+
P. A.	3+	..	..	Observation	Observation
E. B.	..	—	..	Observation	Observation
N. C.	3+	..	..	Observation	Observation
M. D.	+	..	..	Suspicious	Suspicious
L. R.	..	—	..	Observation	—
L. D.	3+	..	..	Observation	Observation
A. R.	..	..	..	Observation	Observation
A. M.	+	—	..	Observation	Observation
A. Mac.	+	..	..	Observation	Observation
J. F.	..	..	±	Observation	Observation
J. V.	2+	..	..	..	Observation
V. M.	3+	..	..	—	Observation
R. Ca.	..	—	..	Observation	Observation
M. H.	2+	..	..	Observation	Observation

In table 5 is given a comparison of the laboratory tests in 43 culturally negative cases.

TABLE 5

RESULTS OF COMPLEMENT FIXATION AND SMEAR EXAMINATIONS IN 41 CULTURALLY
NEGATIVE CASES

Cases	Complement Fixation*			Smears†		Smear at Time of Culture	Culture Material
	Positive	Negative	Doubtful	Urethra	Cervix		
M. K.	..	—	..	Observation	Observation	Observation	Cervix
H. B.	..	..	..	+	—	—	Cervix
M. Kar.	..	..	±	+	—	—	Cervix
M. W.	..	—	..	Suspicious	Observation	—	Cervix
G. B.	2+	..	..	Suspicious	Observation	Observation	Cervix
A. H.	2+	..	..	Suspicious	Observation	Observation	Cervix
B. M.	2+	..	..	+	—	—	Cervix
E. W.	+	..	..	Observation	—	—	Cervix Urethra
M. A.	..	—	..	+	—	—	Cervix
M. S.	4+	..	..	Observation	—	Observation	Cervix
R. G.	+	..	..	Observation	—	—	Cervix
T. F.	2+	..	..	Observation	—	—	Cervix
J. O'C.	..	—	..	Observation	—	—	Cervix
V. H.	3+	..	..	Observation	—	Observation	Cervix
H. Ba.	2+	..	..	Suspicious	—	Observation	Urethra
W. B.	..	—	..	Observation	—	—	Cervix
A. B.	..	—	..	Suspicious	Observation	—	Cervix
M. B.	+	..	..	Observation	—	—	Cervix
C. C.	3+	..	..	Observation	Observation	Observation	Cervix
M. C.	+	..	..	Observation	Observation	Observation	Cervix
M. D.	2+	..	..	+	Observation	—	Cervix
B. E.	2+	..	..	Observation	—	—	Cervix
M. G.	..	—	..	Observation	—	—	Cervix
H. A.	..	—	..	Suspicious	Suspicious	—	Cervix
V. J.	+	..	..	+	Suspicious	Suspicious	Gland
I. L.	3+	..	..	Observation	Suspicious	Suspicious	Cervix
D. M.	+	..	..	Suspicious	Suspicious	—	Cervix
H. P.	+	..	..	Observation	Observation	—	Urethra
F. F.	..	—	..	Observation	Observation	—?	Cervix
I. F.	2+	..	..	+	+	Observation	Cervix
M. A.	2+	..	..	Observation	Observation	—	Cervix Gland
A. E.	..	..	±	Observation	Observation	—	?
V. R.	+	..	..	+	Observation	—	Cervix
J. R.	..	—	..	—	—	—	Cervix
E. C.	2+	..	..	Observation	Observation	Observation	Cervix
H. M.	2+	..	..	Suspicious	Suspicious	?	?
M. M.	..	—	..	Observation	Observation	Observation	Cervix
E. W.	+	..	..	Observation	Observation	—	Cervix Urethra
E. LaR.	2+	..	..	Observation	Observation	—	Cervix
S. G.	..	—	..	Observation	—	Observation	Urethra
B. F.	2+	..	..	—	—	—	Cervix

* Result of fixation at height of reaction is given. From 3 to 23 tests were made on
each case, with an average number of about 8.
† From 3 to 18 smears from each of these cases were examined with an average number
of about 9. The result most suggestive of gonococcus infection is given.

DISCUSSION

The results of this investigation tend to confirm the general impression that neither clinical observations alone, cultural tests, gram-stained smears nor fixation tests, as single methods of diagnosis, can be relied on as guides to diagnoses of actual infection with the gonococcus in cases of suspected chronic gonorrhea in women.

Considering first clinical observations, we find that 9 of our 29 culturally positive cases harbored the gonococcus in the cervix uteri without exhibition on the part of the patients of symptoms definite enough to warrant a clinical diagnosis of gonorrhea. Also 7 other cases, diagnosed as doubtful gonorrhea, which were culturally negative, gave definitely positive complement fixation and two of them also positive smears. Conversely, among 33 cases given a diagnosis of chronic gonorrhea, there were 5 cases in which the clinical diagnosis was supported neither by the cultural test nor by repeated fixations and smears.

Diagnosis by cultural tests would be, of course, the preferred laboratory procedure if it might be carried out with the facility and confidence which that method enjoys as applied, for instance, to the diagnosis of diphtheria. This, however, is at present quite far from being the case. Although our cultural procedures, as applied to the diagnosis of chronic gonorrhea in women, have yielded results which seem perhaps to be better than any reported heretofore, among 41 culturally negative cases (table 5), which were carefully studied, there were 8 cases giving positive smears and 13 other patients who gave a 2 + to 4 + complement fixation. In justice to the cultural method, however, it should be noted that in 6 of these 8 smear-positive cases, the smears from the urethra alone were positive, whereas the material for culture was obtained from the cervix uteri ; also that the results given in table 5 for fixation and smear examinations were the optimal ones of repeated tests, whereas in the great majority of these patients only a single cultural test was made.

As regards diagnosis by means of gram-stained smear examinations our results would seem to indicate that this method would be likely to yield a considerably smaller percentage of definitely positive diagnoses than the cultural methods which we have employed. The relation of the findings with these two methods is summarized on page 151. It is stated there that 19 of the 26 culturally positive cases gave only the observation type of smear and, on the other hand, that many of the culturally negative cases also gave this type of smear. These observation smears,

however, give no more definite information than their name implies, namely, merely denoting the presence of an inflammatory condition in the urethra or cervix uteri, due to some pyogenic organism, quite possibly to the gonococcus. It is not unlikely, in fact, that secondary infections of these localities by streptococci of low grade virulence or other similar organisms persisting after the disappearance of the gonococcus might well be accountable for some of these observation types of smears and hence, if much dependence is placed on this finding, the release of such cases might be delayed longer than is necessary.

As regards the fixation test, there has been included in our list of positive gonococcus diagnoses all of the one-plus or weakly positive reactions. This has been done because in Miss Wilson's two previous studies [5,7] it has been demonstrated that this test is highly specific and that even weakly positive reactions may be considered as diagnostic of a gonorrheal infection. In these studies it is reported:

1. No degree of complement fixation was obtained in cases known as nongonorrheal. Those controls included 345 cases diagnosed as nongonorrheal and 4 normal laboratory workers.

2. The reaction reported as one-plus represents a definitely positive result and has had, in some instances, a comparative reading by a representative of another laboratory of 2-plus or even higher.

3. Some of our one-plus reactions have persisted for from 5 to 11 weeks. These tests were carefully controlled and could not be considered as "doubtful" from the point of view of technic.

4. The clinicians of the Venereal Disease Service of the New York City Health Department, as the result of a long series of observations, have come to regard a one-plus reaction as diagnostic of gonococcus infection. To quote Dr. Barringer's opinion in a recent paper: [*] "Further study of groups of cases with special reference to undetected foci of gonococcus infection may establish the fact that the complement fixation test is the surest means of estimating when a cure has been effected and that we are justified in keeping the patient under treatment as long as these tests remain positive."

It would seem, then, that in the chronic stage of gonorrheal infection and also in the clinically doubtful cases complement-fixation tests, carefully controlled, will give a much higher percentage of positive diagnoses than cultures or smears (table 3) and that this test constitutes at present the simplest and most effective single guide for the control of

[*] N. Y. State Med. Jour., 1922, 22, p. 145.

such cases. On the other hand, it should be noted that some positive cases will be missed if dependence is placed on complement fixation and smear examinations alone. As is shown in table 4, four of the 16 culturally positive cases (25%) gave persistently negative fixation tests and also indefinite smears (observation cases). The clinical diagnosis for 3 of these cases was chronic gonorrhea and for the other "doubtful gonorrhea." Then, too, a persistent one-plus reaction may mean in some cases past rather than present infection with the gonococcus. We do not know how long the antibodies may linger in the body after the period of actual infection has terminated.

<div align="center">SUMMARY</div>

By way of a general conclusion it may be stated that the smear, cultural and complement-fixation methods of diagnosis in chronic gonorrhea in women have all proved useful, and that their relative values correspond to the order in which they are named, the last being the most valuable. Whenever possible, however, each test should be carried out, as it is shown that they tend to supplement each other.

It would seem likely that the cultural methods utilized in this study might find their most useful application, as far as public health work is concerned, in controlling the period of detention of infected women undergoing treatment and also in determining when cases of vulvovaginitis in children may be pronounced cured. With all such patients, of course, local treatments should be stopped at least 4 or 5 days before the cultures are made. In women patients material should be obtained for culture from both the urethra and the cervix uteri. At best it must be admitted that the conditions essential for the isolation of the gonococcus from these chronic infections of women are exacting and can be met only by one experienced in bacteriologic technic and with the facilities of a well-equipped laboratory. In spite of the greatest precautions, too, the plates at times may become overgrown with contaminating bacteria. The method is also more time-consuming than are the smear and complement-fixation procedures. A positive report cannot be made, at the earliest, before 2 days and for a negative report from 4 to 5 days may be required. One the other hand, the isolation of the gonococcus from one of these patients answers the question of infection in an entirely definite way and, under certain conditions, the results obtained may well repay the time and trouble necessary for the application of these cultural procedures.

AN EXPERIMENTAL STUDY OF THE EFFECT OF AUTOGENOUS B. COLI VACCINES ON THE INTESTINAL COLON BACILLI OF DOGS

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From time to time, within recent years, the use of autogenous *B. coli* vaccines has been advocated as a therapeutic measure in the treatment of such conditions as chronic intestinal toxaemia (1) and eczema (2) on the assumption that the toxic substances giving rise to these conditions are produced through the activities of certain *B. coli* vegetating in the intestinal tract, and that these strains may be suppressed or eliminated through specific immunization. Apparently, however, this mode of treatment has not been substantiated by any experimental evidence either that the *B. coli* of the intestinal tract may be controlled through specific therapy or, if particular strains are reduced in numbers through this procedure, that the effect obtained is more than transitory. The study reported here was undertaken with the hope of throwing some light upon these points.

Normal dogs were utilized in these experiments in which an attempt was made to reduce in numbers or eliminate certain strains of *B. coli* naturally vegetating in their intestines.

All organisms belonging in the colon group ferment lactose, but only certain varieties split sucrose. Advantage was taken of these distinctions within the group in selecting strains for the preparation of the vaccine and in estimating the specific effect of inoculation on the distribution of colon types in the fecal specimens. In other words, if there exists any rational basis for attempting to control in a practical way through specific vaccine therapy the types of *B. coli* within the intestinal tract,

then in these experiments inoculation with representatives of the sucrose-positive *B. coli* should cause an elimination or, at least, a marked reduction of these types as revealed in examinations of the fecal specimens.

The dogs, used in these experiments, were kept on a constant diet of boiled rice and boiled beef hearts in the ratio by weight of about 2 to 1. This diet was found by one of us (3) to be favorable for the development of an intestinal flora dominated by *B. coli*. In fact often the only colonies appearing on the Endo plates were *B. coli*-like. The amount of rice and meat fed was not weighed each day, as there would be no advantage in such precautions, but the relative proportions were kept approximately constant.

The fecal specimens were natural movements collected in the morning. Rather heavy emulsions, representative of the whole stool, were made in normal saline solution, and from suitable dilutions Endo plates were seeded. In the preparation of these Endo plates sucrose was substituted for the usual lactose. On these plates, of course, the sucrose fermenting strains of *B. coli* appeared as red colonies, whereas the varieties of *B. coli* which cannot split sucrose gave rise to white colonies. After twenty-four or more hours incubation the ratio of "whites" to "reds" among the *B. coli*-like colonies was determined and recorded. Frequent control tests were made to demonstrate the sucrose fermenting properties of the bacilli forming the red colonies and they were invariably found capable of splitting this sugar. Differential cultural tests were also carried out on a large number of isolated strains to establish their identity. Among the several hundred cultures examined no representative of the *B. aerogenes* type was encountered. Of one hundred sucrose-positive, gelatin-negative cultures from one dog thirty were positive for salicin and seventy were negative. According to Levine's (4) classification of the sucrose fermenters the salicin-positive types should be designated as *B. neapolitanus* and the salicin-negative as *B. communior* or *B. coscoroba* depending upon motility. Among three out of the four dogs differential fermentation tests with the sucrose-positive *B. coli* showed the salicin-negative types

considerably in the majority. Gelatin liquefying strains among the sucrose-positive cultures seemed to be comparatively rare as out of 150 strains examined only 10 liquefied gelatin; accordingly about 7 per cent of these cultures should be placed in the *B. cloacae* group. The main objective in these differential cultural tests was, of course, the selection of sucrose-positive *B. coli* representative of all present in the intestinal flora. All the varieties of sucrose-positive *B. coli* isolated were incorporated in the vaccine except the gelatin liquefiers.

For the purpose of these experiments, then, a definite group of *B. coli*, viz., the sucrose fermenters, was selected to test the practicability of controlling these and related intestinal organisms through specific immunization. These bacilli are constant and normal inhabitants of the intestinal tract and ones which may be recognized readily through differential cultural and serological tests. In some ways it would have been preferable if bacterial species foreign to the intestinal tract could have been utilized. Implantation of such foreign strains, however, does not seem to be possible, and no experiments along that line were attempted.

Preliminary examinations for each animal were made at regular intervals for a period of four to six weeks with the purpose of determining not only the average ratio and the degree of variation in the comparative prevalence of the red and white colon colonies for the normal animal, but also for the selection of representatives of the main cultural variants among the sucrose-positive *B. coli*. With these selected cultures a vaccine was prepared and also rabbits were immunized for the production of a specific anti-serum. When the range in the ratio of the red and white colonies for the normal animal had been determined, a series of vaccine inoculations were given and the cultural examinations were continued at frequent intervals. At each plating during the period of immunization ten well isolated red colonies were transferred to agar slants and agglutination tests were carried out with each, by using the serum from the rabbit inoculated with the vaccine cultures. This anti-serum had a titer of 1-5000 to 1-10,000 for each of the vaccine cultures.

Tests with the isolated strains were made macroscopically at dilutions of 1-50 and 1-500. A sucrose-positive strain was not considered serologically related to the vaccine cultures unless it was definitely agglutinated at the 1-500 dilution. The object of the agglutination tests was to determine whether the sucrose-positive *B. coli* persisting in the intestinal tract in spite of the vaccine treatments were serologically related to the strains used in the vaccine or were entirely distinct strains which had grown up and replaced the originally prominent sucrose-positive *B. coli* types.

As has already been intimated, both the salicin-positive and the salicin-negative representatives of the sucrose-positive *B. coli* were selected in preparing the autogenous vaccine. Agar slant cultures of these strains were emulsified in normal saline and killed by exposure at 60°C. for one hour; the count was determined by the Wright method. The dog inoculations were given subcutaneously generally every two or three days for three or four treatments and then after an interval of a week or more the series of injections were repeated. The dosage in general ranged from one billion to eight billion. Except for a fairly marked loss of weight the animals seemed to suffer no ill effect from the inoculations.

In connection with each of the four experimental animals evidence was produced that treatment with an autogenous *B. coli* vaccine tended to cause a reduction in numbers and, in some instances, an apparently almost complete elimination of the particular varieties of *B. coli* used for immunization. This elimination, however, was more apparent than real; for subsequent examinations would reveal the presence of the same colon types, although it might be in very small numbers. In fact there was no evidence that it was possible, even with very large dosage of antigen to cause their disappearance permanently and completely.

In the tabulation the results for each dog is given. As may be noted, a considerable individual variation was encountered in the readiness and degree to which the animals reacted to the vaccine. Dogs 2 and 3 responded quite readily, whereas num-

bers 1 and 4 were more refractory. With dog 1 a prelim-
inary examination period of five weeks revealed, on the
average, an approximately equal number of red (sucrose-posi-
tive) and white (sucrose-negative) *B. coli* colonies. Following a
billion dosage of the autogenous vaccine, repeated four times,
the relative number of red colonies decreased until a week after
the last injection they were outnumbered by the white colonies,
9 to 1. Soon, however, although an increased dosage was given,
a change occurred in the ratio which would have been misleading
unless agglutination tests had been carried out simultaneously.
Apparently the vaccine had lost its effectiveness in that the
white *B. coli* colonies were outnumbered by the red on the
average 1 to 2. That this inference had no basis in fact was
revealed in an examination of the agglutination results. For,
whereas, in the previous period 91 per cent of the red colonies
surviving the vaccine treatment yielded positive agglutinations
at 1-500 dilution of the anti-serum, now an average of only 26
per cent agglutinated positively; indicating that serologically
unrelated strains of sucrose-positive *B. coli* had grown up and
replaced the original varieties with which the vaccine was pre-
pared. Further examinations indicated that this substitution
was only temporary as at the end of the period of examinations
of this dog 86 per cent of the sucrose-positive *B. coli* aggluti-
nated positively with the specific anti-serum, although on the
average the white colonies now outnumbered the red five to one.
This tendency of serologically unrelated strains of sucrose-
positive *B. coli* to grow up was noted also with dogs 2 and 3,
appearing in each instance at about the same stage of the im-
munization process, but in no case could these substituting
strains maintain themselves.

In experiments of this character it is important to carry out a
long series of preliminary observations to determine the degree
to which the types of *B. coli* selected vary in numbers under
ordinary conditions. A period of six to eight weeks was con-
sidered adequate for this purpose. After inoculations were
started about two to three weeks elapsed before definite results
were noted in the differential *B. coli* determinations. In dog 3

an apparent rapid elimination of sucrose-positive strains occurred within a week after the first treatment with the vaccine. It is questionable, however, if this change may be ascribed to the specific action of the vaccine. As regards dosage it was found than an inoculation with about 2 to 4 billion *B. coli* secured the maximum effect, and that nothing was gained by increasing the amount above this point. In one experiment a dosage as small as 100 million was employed. After five inoculations covering a period of three weeks there seemed to be some slight effect, but not as definite as following the larger dosage.

Observations were not continued long enough to determine definitely how enduring the effect of the inoculations might be. In the case of one animal, dog 2, the effect was undiminished after the lapse of ten weeks following the last of a series of six inoculations. With dog 3 the effect seemed to have largely worn off in seventeen weeks, but during the interval the diet had been somewhat changed. Diet, in fact, is the factor of prime importance in the determination of the types of bacteria pullulating in the intestinal tract, and without an uniform diet the results of these experiments would have been indefinite and misleading. An experiment with dog 4 showed how readily modification of the diet would upset the relative distribution of *B. coli* types established through vaccine inoculations. A week after the animal had completed the immunization process and the sucrose-negative *B. coli* had become predominant, fifty grams of lactose was added to the diet of rice and meat. Within three days there was noted a very marked increase in the *B. coli* count with about an equal number of red and white *B. coli* colonies on the Endo plates. This apparent inhibition of the specific repressive action of *B. coli* inoculations continued as long as lactose was a part of the diet.

Although the question of the relative effect of autogenous and heterogenous vaccines was not made a feature of this study, yet some experiments with dog 2 indicated that to obtain marked results it was necessary to use an autogenous vaccine. After a preliminary observation period of six weeks, this animal was given three weekly inoculations of the *B. coli* vaccine prepared

from cultures derived from dog 1 in a dosage of two billion. Very little in the way of definite effect, however, was noted until an autogenous vaccine was used when a marked and persistent decrease in the relative numbers of the sucrose-positive B. coli followed.

With one animal (dog 4) an attempt was made to correlate the cultural results with the rise of antibody (agglutinin) in the blood following the inoculations. The serum from this animal before inoculation agglutinated an autogenous sucrose-positive B. coli culture at 1-160 dilution. This amount of agglutinin showed first a definite enhancement about two weeks after a series of three inoculations had been given, when the titer rose to 1-640. Shortly after this increase in anti-body content of the blood, a definite decrease occurred in the number of sucrose-positive B. coli in the fecal specimens examined. Continued inoculations with larger doses of the vaccine finally sent the titer up to 1-1280, with a coincident repression of the sucrose-positive B. coli as shown in the tabulation. A number of agglutination tests with extracts of the fecal matter were carried out to determine to what extent this antibody found its way into the intestinal tract. Positive results were obtained with watery defecations and especially with saline extracts of the mucus, but not with extracts from emulsions of the formed or semi-formed matter.

These experiments with dogs have established the fact that it is possible through the use of autogenous vaccines to effect at least a temporary suppression of corresponding strains of B. coli naturally vegetating in the intestinal tract. Assuming that some varieties of B. coli may be of importance in connection with intestinal toxaemia, a certain amount of experimental justification is accorded the employment of an autogenous vaccine therapy. In fact it should be much easier to effect by this means the suppression of parasitized B. coli than of the normal vegetative types which are firmly established in the intestinal tract. Such being the case one of the real difficulties in applying this form of therapy is to determine just what strains of B. coli may be giving rise to toxic products and to incorporate these in

TABLE 1

Effect of inoculation with B. coli vaccine on the types of B. coli vegetating within the intestines of dogs

DOG	DATES OF EXAMINATIONS	DATES AND DOSAGE OF VACCINATION	AVERAGE RATIO OF SUCROSE-NEGATIVE TO SUCROSE-POSITIVE B. COLI	PER CENT OF SUCROSE-POSITIVE B. COLI AGGLUTINATING WITH ANTI-SERUM TO THE VACCINE CULTURES	REMARKS
1	XI-12, 19; XII-10, 12, 16, 20, 1918		1-1		Pre-vaccination period
1	XII-28, 31, 1918; 1, 3, 4, 8, 1919	XII-21, 1 B. XII-28, 1 B. XII-30, 1 B. I- 2, 1 B.	3-1		Vaccination period
1	I-14, 20, 22, 1919		9-1	91	
1	I-24, 27, 30; II-2, 4, 6, 1919	I-24, 3 B. I-28, 3 B. I-30, 3 B.	1-2	28	Increase in number of other strains of sucrose-positive B. coli
1	II-9, 11, 13, 15, 17, 1919		2½-1	58	
1	II-25, 27; III-3, 5, 7, 1919	II-20, 4 B. II-26, 4 B.	5-1	86	Above strains have decreased in numbers
2	I-9, 14, 16, 20, 22, 24, 27, 30, 1919		1-3	94	Pre-vaccination period
2	II-2, 4, 6, 9, 11, 13, 17, 19, 1919		2-1	81	Pre-vaccination period
2	II-25, 27; III-3, 5, 7, 12, 1919	II-20, 2 B., Dog I vaccine II-26, 2 B., same III-5, 2 B., same	2-1	91	Heterogenous vaccine

No.	Dates	Injections	Sucrose ratio	Per cent	Remarks
2	III-15, 18, 21, 26, 1919	III-13, 2 B., Dog II vaccine; III-19, 4 B., same; III-24, 4 B., same	1-3	39	Autogenous vaccine
2	III-28; IV-1, 3, 7, 9, 1919		All sucrose-negative; 6-1	28	Apparent elimination of sucrose-positive B. coli
2	IV-11, 14, 1919				
2	IV-17, 21, 23, 25; V-2, 5, 8, 12, 1919		4-1	71	
2	V-15, 1919		All sucrose-negative; 18-1	17	
2	V-19, 21, 23, 26, 28, 31, 1919		12-1	83	
2	VI-4, 6, 9, 1919	VI-10, 8 B.; VI-16, 8 B.; VI-18, 8 B.	13-1	80 (2 tests)	Large dosage of vaccine
2	VI-11, 13, 16, 18, 1919		7-1	13 (3 tests)	Pre-vaccination period
3	VI-20, 23, 27, 30 1919		12-1	100	
3	III-12, 14, 1919				
3	III-18, 1919				
3	III-21, 26, 28; IV-1, 1919		All sucrose-positive; 11-1	90	
3	IV-3, 1919				
3	IV-7, 9, 11, 17; 21, 23, 25, 1919		All sucrose-positive; 5-1	70	Vaccination period. Autogenous vaccine
3	IV-29; V-2, 5, 8, 1919	V-9, 2 B.	2-1	95	
3	V-12, 1919		90-1	0	
3	V-15, 1919				
3	V-19, 21, 23, 26; 28, 31, 1919	V-20, 2 B.; V-27, 4 B.	All sucrose-negative; 6-1	45	

DOG	DATES OF EXAMINATIONS	DATES AND DOSAGE OF VACCINATION	AVERAGE RATIO OF SUCROSE-NEGATIVE TO SUCROSE-POSITIVE B. COLI	PER CENT OF SUCROSE-POSITIVE B. COLI AGGLUTINATING WITH ANTI-SERUM TO THE VACCINE CULTURES	REMARKS
3	VI-3, 1919		All sucrose-negative		
3	VI-4, 6, 9, 11, 13, 1919	VI-6, 4 B.	16-1	64	
3	VI-16, 18, 20, 23, 25, 27, 30; VII-2, 1919		31-1	32 (average of 5 examinations)	
3	VII-5, 1919		All sucrose-negative		Pre-vaccination period
3	VII-7, 9, 11, 1919		25-1		
4	VIII-12, 14, 21, 26, 29; IX-3, 8, 12, 1919		17-1	30	
4	IX-18, 22, 29; X-3, 6, 9, 1919		5-1		
4	X-13, 15, 16, 21, 23, 25, 1919	X-14, 3 B. X-17, 3 B. X-20, 3 B.	1-3		Vaccination period. Autogenous vaccine
4	X-27, 29, 31; XI-3, 5, 1919	X-27, 6 B.	44-1		
4	XI-7, 10, 13, 14, 17, 1919	XI-1, 6 B. XI-8, 6 B.	28-1		
4	XI-24, 26, 28; XII-2, 4, 1919	XI-15, 6 B.	2-1		Lactose (50 grams) added to diet November 18, and after

the vaccine.   Apparently heretofore vaccines of this character have been made up in a rather hit or miss fashion.   It should also be borne in mind that diet is the most important factor in the regulation of the intestinal flora.   In fact these experiments indicate that an attempt to control types of bacteria germinating in the intestinal tract through specific vaccine therapy is futile unless the diet is also carefully regulated.

CONCLUSIONS

1. In the case of a number of dogs it has been found possible to effect the temporary suppression of a certain variety of *B. coli* normal to the intestinal tract through inoculations with a specific vaccine.

2. Autogenous vaccines are apparently necessary for marked results and the dosage must be large.

3. Cultural results indicating a decrease in numbers of the type of *B. coli* in question were associated with a coincident rise of specific anti-body in the blood.

4. A uniform diet must be maintained, otherwise the effect of the vaccine will be obscured.

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A CULTURAL STUDY OF ANAEROBIC SPORE–BEARING BACTERIA WITH STRAINS ISOLATED BY THE BARBER SINGLE CELL TECHNIC *

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Everyone familiar with the literature concerning anaerobic spore-bearing bacteria doubtless has been impressed with the dissimilarity of the cultural reactions as reported by various observers. Aside from faulty technic and a seeming zeal on the part of many investigators to discover new species within this complicated group, impurity of culture seems to be responsible for much of the chaotic condition in which we now find a large part of the subject. One of the outstanding sources of error as regards impurity of culture is the difficulty often encountered when an attempt is made to obtain a pure culture from a single surface or deep colony. This point has been brought out by Hall [3] and others but little notice seems to have been taken of it. I have found on numerous occasions that a single colony of a supposedly pure culture of B. welchii, for instance, may be planted into media of differential value and after twenty-four or forty-eight hours incubation give rise to the usual typical reaction. But often upon standing in the laboratory for a few days, proteolytic activities suddenly make their appearance. This secondary action of a so-called proteolytic ferment in the case of B. welchii seems to have been noted by so many observers, that it is often considered one of the cultural characteristics of the organism. I have found, however, that the strains subjected to isolation by the Barber technic [41] did not show the slightest evidence of proteolysis even over a period of eight months.

In a study, now in course of preparation, on the types of anaerobic spore bearers occurring in human feces it has often

been my experience that a single colony, even as occurring in deep shake culture, may be composed of individuals of more than one species and for this reason the dilution method has not been wholly relied upon as a means of obtaining pure culture. However, the dilution method of Veillon and Zuber [20] when supplemented by reisolation with the Barber single cell apparatus has been found to be wholly reliable for obtaining those organisms in pure condition.

· The world war stimulated much study on anaerobic bacteria and during the past few years many valuable contributions have been made. At final analysis, however, none of the cultures used by the various observers would seem entirely free from the possibility of impurity.

It has been the writer's good fortune to come into the possession of a collection of fifteen anaerobic spore-bearing bacteria of different species whose origin and purity of culture seems beyond question and it is the purpose of this paper to record in detail the results of cultural observations made upon these organisms. A review of the literature has impressed the writer with the fact that a large number of observers have failed to publish in sufficient detail the methods employed, contenting themselves with recording their results and leaving the technic by which such results were obtained largely to the reader's imagination. Care, has, therefore, been taken to trace the course of this study step by step.

The literature having been but recently quite thoroughly reviewed by Heller [4, 10, 11], no attempt has been made to go into the matter here.

My observations are at variance with those made by Robertson,[9] Henry,[14] and others regarding the value of methods of differentiation through colony morphology. As will presently be seen, methods for obtaining surface growth were found to be unreliable, and the colonies in deep culture were found to vary so much under conditions of differences in moisture, type of media, age of culture, and hydrogen ion concentration of the media, that no emphasis has been placed upon this as a point of differential value. Even when organisms were planted upon the same medium, specific morphological differences were in

the main found to be slight and with the exception of one or two species gave no aid in telling one species from another.

The following organisms were used in this study:

Strains used

B. sporogenes (Metchnikoff) [22] 1
B. histolyticus (Weinberg) [30] 2
B. oedematis maligni (Koch) [19] 1
B. botulinus (van Ermengen) [33] 1
B. putrificus (Bienstock) [31] 3
B. tetani (Kitasato) [35] (Nicolair) [36] 3
B. bifermentans (Tisser and Martelly) [34] 1
B. bellonensis (Sacquepee and Veillon) [32] 1
B. aerofoetidis (Wienberg) [28] 1
B. oedematiens (Wienberg) [29] 1
Vibrion septique (Pasteur) [38] 1
B. of Ghon and Sachs (Ghon and Sachs) [37] 1
B. fallax (Weinberg) [27] 1
B. welchii (Welch and Nuttall) [23] 4
B. tertius (Henry),[14] (Flemming),[24] (Rodella),[15] (Robertson),[9] (von Hibler) [26] 2

The writer obtained the entire collection from Dr. M. A. Barber and they were used by Dr. Barber [6] in his investigation upon the use of the single cell apparatus in isolating anaerobic bacteria in pure culture. The cultures of B. sporogenes, B. histolyticus, B. putrificus, B. oedematis maligni, B. bifermentans, B. bellonensis, B. aerofoetidis, B. oedematiens, Vibrion septique, B. of Ghon and Sachs, B. fallax, and B. tertius were obtained primarily by Dr. Benjamin Jablons (late Major, M. C., U. S. A.) directly from Dr. Weinberg of the Pasteur Institute, Paris. As may be noted from the list, Weinberg originally isolated and described a considerable number of the organisms here studied, and as these particular strains were obtained from his laboratory there can be no question as to their identity with the original Weinberg types. The bacteria obtained from the Pasteur Institute were isolated from infected war wounds.

Duplicate cultures of the organisms listed were obtained from other sources from time to time. These were subjected to the single cell method of isolation at first by Dr. Barber, and later by the writer.

Of the original collection it may be said that they were not even transplants of cultures of single cell parentage, but the actual single organism seeded into suitable culture media. Thus the purity and authenticity of these strains seems beyond question.

Since the results herein set down were obtained from the study of one or at most four separate cultures of the different species, no claims are made to the effect that these cultural reactions would be exactly constant for all representatives of a given species.

Hort [12] has criticized the Barber single cell technic on the following grounds:

1. Once the pipette has been removed, re-examination to determine whether more than one organism is or is not present, is impossible.
2. Control observations of development from single cell to single colony cannot be carried out owing to the exclusive use of liquid media in the early stages.
3. The optical difficulties attendant on the examination of droplets.

The first objection is obscure but in answer I would say that the droplet containing the single microörganism may be thoroughly examined for evidence of a second organism by the high power or oil immersion lens *before* the pipette containing the single cell is removed. In fact, the operator may with ease watch the single bacillus, droplet and all, flow into the waiting capillary tube. No re-examination would seem to be called for unless it be of the late seat of the droplet, to see whether or not the bacillus has entered the pipette. It is true that at times a spore will tenaciously cling to the cover slip and refuse to enter the waiting pipette until a considerable degree of suction has been applied. Such instances are relatively rare and a second examination of the droplet will determine whether or not the organism has been dislodged.

As to the second criticism, it is a relatively simple matter to cultivate a colony of an anaerobic organism from a single cell, *under observation*. The method is as follows: After the droplet containing the single cell has been sucked into the pipette, instead of removing the capillary tube from the pipette holder it is so manipulated as to bring it under a droplet of nutrient agar

of $\frac{1}{2}$ or $\frac{3}{4}$ per cent. A small amount of this is sucked up into the pipette and the small glass hook containing the single cell is broken off into the residual portion of the agar droplet by bringing pressure to bear with the vertical control screw. The agar hardens and forms rather an effective seal. In this manner a single cell of an anaerobic organism may be frequently observed to grow into a single colony.

The third objection was answered by Barber [6] and the writer can do no better than to quote him to the effect that one is able to see any organism in the droplet visible to the microscope, and further, that the drop is not large and rounded so as to hide an organism in its depths, but flat and small; thus all parts are easily visible.

The unfavorable results which some investigators have had with this apparatus are no doubt due, in part at least, to the fact that the method is difficult to learn from a reading of the literature. There are numerous minor details of operation which are only to be gathered by watching the actual manipulation of the apparatus, or better yet by operating it oneself under the guidance of someone familiar with it.

The Barber method of isolation proved entirely satisfactory for the purpose of this study, so much so, in fact, that it would seem to be the most feasible and satisfactory way of obtaining anaerobic spore-bearing bacteria in pure culture.

As to a preference for transplanting single spores rather than single bacilli when using the single cell method of isolation, my results agree with those reported by Barber [6] who found that single spores give by far the largest number of successful cultures. Such organisms as tenaciously refuse to sporulate upon ordinary laboratory media may be induced to do so by planting them upon a subsequently described sugar free nutrient substance. (See morphological descriptions.)

Stock Cultures. — The entire collection of stock cultures was kept alive upon one-half per cent of beef infusion agar to which had been added two per cent of Wolf's casein digest substance (see fermentation tests for formula). Animal serum so widely used by many observers as an enriching agent was

not employed. The casein digest solution was found to be as favorable as serum and has the added advantage of being sterilizable by autoclaving without effecting its growth stimulating properties. It was found unnecessary to add any carbohydrate to the stock media. Anaerobiosis was induced by the vaseline cap boiling method to be described in detail later. The media was at all times adjusted to the hydrogen Ion concentration of Ph. 7.2. This was found to be the most favorable point of growth for all of the test organisms. My results here agree with those obtained by Dernby and Blank.[13]

It would seem that much unnecessary attention has been paid to devising elaborate formulae for media upon which to induce these organisms to grow, while the fact is that they multiply rapidly and grow profusely in comparatively simple substances if the hydrogen-ion concentration is adjusted properly and adequate anaerobiosis is obtained.

The cover of vaseline has the advantage of affording excellent anaerobiosis, and also prevents evaporation. One set of cultures kept in the ice-box were found alive after two years and upon transplanting invariably gave rise to luxuriantly growing sub-cultures.

As most of the organisms are able to ferment muscle sugar, the cover of vaseline is slightly lifted from the surface of the media through the pressure of the gas formed. It is a wise precaution, accordingly, after twenty-four hours incubation to gently heat the vaseline cap in the Bunsen burner and allow it to again come in contact with the media. It is well to repeat this procedure after forty-eight hours have elapsed. The little gas accumulated after this time is not enough to give rise to further difficulty. Unless double the usual amount of vaseline is added to the cultures of B. welchii, they will invariably blow the cotton stopper as well as the vaseline entirely from the tube. The weight of an additional amount seems sufficient to prevent this.

As a matter of routine the organisms were planted upon fresh media every three weeks and as an added precaution the entire collection was subjected to the single cell method of isolation each six months.

From time to time cultural tests were made upon all organisms to detect possible aerobic contamination. None, however, occurred during the entire course of the study and it seems quite evident that if the usual precautions of technic and sterility are observed, one may expect no more difficulty in this regard than is experienced with aerobic bacteria.

Methods. — Before attempting this investigation, several months were devoted to a study of the methods used in the cultivation of anaerobic bacteria.

Bulloch's jar, Novy's apparatus, Buchner's tube, and a number of other methods, for the most part modifications of the above, were given a thorough trial. Vacuum exhaustion, as well as the displacement of oxygen with hydrogen, was also tried. On the whole these methods were uncertain as regards results when surface growth or growth in liquid media was desired. Some of the organisms not over fastidious in their anaerobic requirements did grow with a fair degree of regularity. But when attempts were made to cultivate anaerobes more exacting in their oxygen requirements, such as B. histolyticus or Vibrion septique, negative results were obtained in such a large number of cases that the above technics, together with their various modifications, were discarded as unsatisfactory for the purposes of a systematic study of the biological characteristics of a variety of bacteria, differing, it is true, in their degree of *necessary* anaerobiosis, but all obligate anaerobes.

The methods of Laidlaw,[1] McLeod and Saga,[2] and others of similar nature involving oxygen displacement with hydrogen, and an additional precaution of having suspended in the container a fragment of platinized carbon, or kindred substance, were thoroughly investigated. The platinized carbon glows dull red as soon as it is introduced into the jar and is supposed to continue to do so until all of the residual oxygen is used up in forming water; the theory being that as the oxygen is given off, it is absorbed by the platinum and burned to water. These methods seemed feasible because of their simplicity and the chemically sound principles upon which they are based.

Every step as stated by the various authors was followed with
scrupulous care, but in my hands the technics involving the
above-mentioned principles proved even less satisfactory than
the use of pyrogallic acid and sodium hydroxide. Various
modifications and combinations of the pyrogallic acid, hydro-
gen displacement, vacuum exhaustion and platinized carbon
procedures were tried with little or no better success. Finally,
following Hall,[3] the deep-culture methods of the French ob-
servers, Veillon and Zuber, were used with greater success.
Growth was obtained in a large majority of cases. But some
of the more exacting organisms such as B. aerofoetidid, B.
oedematiens, B. bellonensis, and others, gave a large per-
centage of negative cultures. When growth, especially in
liquid media was desired, no aid could be obtained from the
so-called French deep culture methods. Sterile paraffin oil
(albolene, liquid petrolatum) was applied to the top of the
media — improving the results to some extent. However, it
was not until caps of petrolatum jelly, 1.5 cm. or more in
height, were placed upon the tops of the culture media (which
had previously been boiled for fifteen minutes) that growth
was obtained with unfailing regularity. This method was
first employed by Loewe and Strauss [5] and soon after by Bar-
ber.[6] The hard paraffin method of Thompson [7] gave results
equal to those obtained with vaseline. It consists in simply
substituting a cap of paraffin with a melting point of 55° C. for
the petrolatum jelly. Thompson's method was finally aban-
doned in favor of the method of Loewe and Strauss, as the
vaseline cap is much more easily manipulated when one desires
to transfer or examine cultures.

That the vaseline cap is far superior to liquid petrolatum as
a method of obtaining anaerobiosis, was clearly shown by
Gates and Olitzsky.[8] These observers used methylene blue as
an indicator of relative anaerobiosis. Before knowing of their
work the author undertook to investigate this point using the
actual organisms. The entire collection of anaerobes was
planted in beef infusion casein digest broth and anaerobiosis
was induced with liquid paraffin oil. Growth took place only
in the tubes containing B. welchii, B. oedematis, B. sporogenes,

and slightly in the tube containing B. botulinus, as observed after twenty-four hours incubation. The usual amount of vaseline was then placed in each tube without disturbing the oil, after which the cultures were returned to the incubator for a second twenty-four hours. Growth occurred luxuriantly in the case of everyone of the organisms. A control series grown under vaseline upon the same medium gave profuse growth in every culture after the initial twenty-four hour incubation period.

In practically all experiments performed, the media, the solid, semi-solid or liquid was tubed in 10 c.c. amounts in test tubes 6″ × ⅞″. Just prior to inoculation, the media are thoroughly boiled for fifteen minutes to expel as much of the dissolved oxygen as possible and then rapidly cooled to about 40° C. Inoculations were made and to each culture a cap of sterile vaseline was applied about 1.5 cm. in height. This was done, of course, with a sterile pipette. Just before seeding the vaseline was sterilized in the autoclave for the usual length of time. The tubes were then rapidly cooled to secure quick hardening of the vaseline. In this way nearly perfect anaerobiosis was obtained.

A series of experiments was performed to determine whether or not it was necessary to apply the cap of vaseline to the agar before the inoculation was made.

Two sets of broth cultures were used. One set had the vaseline applied before seeding and was inoculated while the vaseline was still in liquid form, and to the other set (the entire collection of fifteen anaerobes of different species was used) the vaseline was added after inoculation. Growth was equally luxurious in both cases, so that the former procedure, i. e., inoculating through the vaseline cap, was discarded as unnecessarily laborious.

The method of growing anaerobic bacteria as described above was by far the most successful, because:

1. Growth is obtained in the maximum number of cases, showing excellent anaerobiosis.
2. The method is simpler and less time consuming than any other.
3. It is the least expensive.

4. It is applicable to any form of media including liquid — where surface growth is not desired.

5. When fermentation tests are performed, an accurate idea of gas production may be had from the height to which the vaseline cap is forced in the tube, which at the same time remains air tight. This is far superior to the small Dunham tube method of gas measurement (see fermentation reactions).

Use of the Pasteur Pipette. — Due to the use of the vaseline cover it was quite impossible to employ the platinum loop for making transfers and manipulating the organisms. The Pasteur pipette was on the other hand found to be quite indispensable.

Soft glass tubing, $\frac{1}{4}$ inch in diameter, is cut into six-inch lengths, plugged with cotton at either end and sterilized in the hot air sterilizer. The tube just prior to use is applied to the flame of an ordinary laboratory Bunsen burner and slanted in such a way that the maximum portion of the glass is covered by the flame, care being taken to rotate the tube constantly to insure equal application of the heat, When a dull red color appears and the glass bends at slight pressure it is quickly removed from the flame and drawn out gently and gradually, taking care not to make the pulling jerky. Thus, one obtains two separate pipettes. (A small wire basket, sterilized by passing the flame of the burner over it a few times, serves as an excellent rack upon which to rest them.) Just prior to use, the fine end of the pipette is applied to the somewhat cooler, lower, outer edge of the flame and redrawn into a still finer portion. This is sealed by giving it a second cautious application of the burner. The method of use is to plunge the pipette through the cover of hardened vaseline and by gently tapping the end against the bottom of the test tube the seal is broken. By gentle suction, enough of the culture is drawn up to serve any purpose. Isolated deep colonies may be successfully fished in this manner. Only a small hole is made in the vaseline cap and the culture may be immediately sealed by slightly warming the top of the vaseline.

Saccharolytic Reactions. — Probably the most comprehensive attempt to classify the anaerobes according to their

carbohydrate splitting properties was made by Henry,[14] 1917. His work throws light upon many important points, but the writer feels that the methods employed are open to criticism.

1. Method of obtaining anaerobiosis. — As a preventive to the influx of atmospheric oxygen, Henry covered the cultures with paraffin oil, after the media had been thoroughly boiled. As has already been pointed out, Gates and Olitzsky found that paraffin oil not only did not act as an effective oxygen barrier, but actually acted as a reservoir for it. Paraffin oil may serve quite effectively as a method of obtaining anaerobiosis when one or two per cent agar is used as a base for media, for, as is well known, agar of this consistency acts as a fairly effective oxygen barrier in itself. In fact some of the organisms studied will multiply feebly in the depths of stiff agar tubes without either the presence of vaseline or paraffin. After twenty-four hours incubation oxygen will be found to have permeated the top half-inch or so of the medium, without disturbing the favorable conditions in the depths of the tube. However, when broth or soft agar cultures are used, as they must be in the determination of the presence of sugar splitting ferments, it is absolutely necessary to prevent atmospheric oxygen from intruding. To accomplish this, anaerobiosis in the case of my own tests was obtained by the vaseline boiling method, already described, i. e., substituting vaseline for the paraffin oil. Because liquid media are highly permeable to oxygen, inoculations in these fermentation tests were made through the covers of vaseline while they were still in liquid condition. As an added precaution great care was exercised not to empty the full content of the Pasteur pipette into the medium when inoculations were being made. For when this did occur, profuse bubbling invariably took place. After seeding, the tubes were quickly cooled to insure rapid hardening of the vaseline.

It is felt that in this way anaerobiosis was more effectively procured than by the methods used by Henry and thus the conditions were more favorable for the action of the sugar splitting ferments.

2. Media for saccharolytic tests. — A suitable substance upon which to grow these organisms in sugar-free liquid culture was not easy to find. Henry found ordinary beef infusion broth unsuitable for this purpose. This was also my experience, several of the more fastidious organisms refusing to multiply at all. Henry subsequently recommended the use of the neutral pancreatic digest substance, made for him by Captain Wolf, in dilution of one part casein digest fluid to two parts of physiological salt solution.

Wolf's casein digest substance was found to be an enriching agent of extraordinary merit which, when added to broth or agar in quantities of two per cent, did away with the necessity for using animal serum, egg fluid, ascitic fluid or substances of a similar nature. It has the added advantage of not having its nutrient principle affected by autoclaving. All of the anaerobes in my collection grew luxuriantly in its presence.

As casein digest fluid is not at present widely used, the formula for its preparation as given by Henry will be repeated here.

To a 2 liter flask containing 1 liter of tap water, add 20 grams of anhydrous sodium carbonate. Transfer the flask when boiling to a water bath and add 200 grams of casein, dusting it in, very gradually, and shaking vigorously from time to time to avoid the formation of lumps. Then place the mixture in a large Winchester quart bottle (I find the original flask to serve the purpose) and allow the substance to cool down and add 3 grams of pancreatin with 15 c.c. of chloroform. Incubate for five days .at 38° C., shaking vigorously from day to day to break up the crusts which have formed. Then add 3 grams more of pancreatin and incubate for 10 days longer. At the end of fifteen days treat the mixture with 400 c.c. of N/1 HCl, steam for thirty minutes to drive off the chloroform and filter. The filtrate is treated with 120 c.c. of N/1 NaOH and the reaction adjusted to Ph. 7.2. This is the stock solution to be added to agar or broth in amounts of 2 per cent.

When the formula for sugar-free broth as recommended by Henry was used, namely, one part casein digest fluid to two parts normal physiological salt solution, it was found that a number of organisms comprised in this collection namely, B. oedimatiens, B. histolyticus, B. aerofoetidis, Vibrion septique, and B. bellonensis, did not grow at all. In the case of other organisms some of the cultures gave evidence of violent fermentation, showing, of course, that the media was not sugar free. This gave rise to the study of a number of substances as possible media upon which to grow the organisms during the saccharolytic tests. Sugar-free beef and veal-infusion broth,

gelatin, Dunham's solution, the egg fluid of Robertson (see proteolytic tests) and Hiss's serum water were all investigated in various combination and dilutions. All were discarded as unsuited for the purpose, since invariably some of the types refused to grow.

The following simple formula was used and found to satisfy all needs. Representatives of the entire collection grew profusely in it and it was readily prepared and easily rendered sugar free.

Beef-infusion broth made in the usual manner is adjusted to Ph. 7.00. To it is then added 2 per cent of the casein digest substance. The broth is seeded with a strain of B. coli which actively ferments dextrose and the whole is allowed to incubate for twenty-four hours. The broth is heated in the Arnold sterilizer for forty-five minutes, and adjusted to Ph. 7.2 by the addition of N/1 NaOH, reheated in the Arnold for fifteen minutes and filtered. It is then tubed in 9 c.c. amounts and sterilized in the autoclave. This medium was tested for sugar free properties by inoculating individuals of the entire collection into tubes containing it. The few bubbles of gas which at times appeared in the cultures of B. sporogenes, B. oedimatis and B. botulinus were felt to be due to proteolytic activities. None of the others gave evidence of the presence of carbohydrates.

The sugars were prepared in ten per cent dilution in small flasks containing the sterile sugar-free medium. They were then heated in the Arnold at 100° for ten minutes. One c.c. of this ten per cent solution of sugar was then added to tubes containing nine c.c. of sterile media, thus obtaining a final carbohydrate concentration of one per cent. The tubes were then capped with sterile vaseline and returned to the Arnold for ten minutes additional sterilization. Inoculations were made with sterile Pasteur pipettes through the cover of liquid vaseline and as each tube received its seeding it was immersed in cold water to insure rapid hardening of the vaseline. As an added and probably unnecessary precaution, to preclude the possibility of sugar being taken over, the stock cultures from which the inoculum was taken were grown upon sugar-free casein digest, fluid agar (see morphological descriptions). The cultures were incubated for 48 hours at 37°C., after which time readings were taken.

3. Gas and acid production. — Henry's method of measuring gas production, namely, through the use of small Dunham tubes placed in broth cultures, seemed to me somewhat inaccurate as in my experience the bubbles of gas escape through the paraffin oil covers and only a few of them, sometimes none at all, are imprisoned in the small Dunham tube, thus often giving rise to erroneous measurements of gas. As has already

TABLE I. FERMENTATION TESTS OF SPORE-BEARING ANAEROBES

Organisms	Glucose		Levulose		Galactose		Saccharose		Maltose		Lactose		Raffinose		Mannose	
	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion
B. sporogenes....	2	5.6	1	5.2	0	5.2	0	6.6	2	5.4	1	6.8	1	6.6	0	7.2
B. botulinus	1	6.8	1	6.8	1	6.8	0	7.2	0	7.2	0	7.2	0	7.2	0	7.2
B. oedematis	2	5.6	1	5.6	1	5.6	1	6.8	0	5.4	0	6.4	1	6.6	1	6.8
B. histolyticus ..	0	7.2	0	7.2	0	7.2	0	7.2	0	6.6	0	6.6	0	7.2	0	7.2
B. putrificus	1	6.8	1	6.8	1	6.8	1	6.8	2	5.0	0	6.4	1	6.7	1	6.8
B. tetani	1	5.8	1	6.6	1	6.8	0	4.8	0	4.4	0	4.6	0	5.0	0	5.4
B. bifermentans..	2	5.6	1	5.0	0	6.5	0	6.5	1	5.0	0	6.8	0	6.4	3	4.8
B. bellonensis....	2	6.0	0	5.8	0	6.2	0	6.6	3	4.6	0	6.6	1	6.4	0	7.2
B. aerofoetidis ..	2	5.4	2	5.0	2	5.0	0	7.2	3	4.2	2	5.0	0	6.4	3	4.4
B. oedematiens ..	2	5.4	1	5.6	2	4.8	2	4.8	3	4.6	2	5.4	2	5.4	3	5.4
B. Ghon-Sachs...	2	5.6	2	4.8	2	4.8	2	5.0	3	4.4	3	4.2	3	5.0	4	4.8
Vibrion sept.	2	5.4	2	4.6	2	5.2	2	5.0	3	4.4	3	4.2	3	4.8	4	5.0
B. fallax	1	5.0	1	5.2	1	5.2	2	4.6	2	5.0	3	4.4	3	4.0	1	6.6
B. welchii (4) ...	4	4.2	4	4.6	3	4.8	4	4.2	4	4.2	4	4.0	3	4.0	4	4.4
B. welchii (2) ...	4	4.6	4	4.6	1	5.2	4	4.4	3	4.6	3	4.6	3	4.6	4	4.6
B. tertius	2	6.4	2	6.4	1	6.6	1	7.0	2	6.6	2	6.6	1	6.6	1	6.6

The numbers in the column marked "gas" indicate the height measured in centimeters to which the vaseline cap had been raised.

TABLE I. FERMENTATION TESTS OF SPORE-BEARING ANAEROBES

Xylose		Arabinose		Starch		Inulin		Dextrin		Salicin		Mannite		Melezitose		Glycerine		Inosite	
Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion	Gas	H-ion
0	6.6	0	7.2	0	7.2	0	7.2	0	7.1	0	7.2	0	7.2	0	7.0	2	6.6	0	7.2
0	7.2	0	7.2	0	7.1	0	7.0	0	7.2	0	7.2	0	7.2	1	6.9	2	6.6	0	7.2
0	6.6	0	7.2	0	7.1	0	7.2	0	7.2	0	7.1	0	7.1	0	7.2	0	7.1	0	7.2
0	6.6	0	7.2	0	7.2	0	6.4	0	7.2	0	7.1	0	7.2	0	7.0	0	7.2	0	7.1
1	6.6	0	7.2	0	7.0	0	7.0	0	7.1	0	7.2	0	7.1	1	6.8	1	6.9	0	7.1
0	5.6	0	7.0	0	7.0	1	6.8	0	7.1	0	7.2	0	7.2	0	7.2	0	7.0	2	6.8
0	6.4	0	7.1	0	7.0	0	7.2	0	7.2	0	7.0	0	7.1	0	7.2	2	6.6	0	7.2
1	6.8	0	6.6	0	7.2	0	7.2	0	7.2	0	7.2	0	7.2	0	7.2	0	6.8	0	7.2
2	5.6	0	7.0	0	6.8	0	6.8	0	6.8	1	6.6	0	7.2	0	7.0.	0	7.0	0	7.2
1	6.6	0	7.1	4	5.8	0	6.6	0	7.2	0	7.2	0	7.2	0	7.1	0	7.2	0	7.1
2	6.6	0	7.2	1	6.8	0	6.6	0	7.0	1	6.6	0	7.2	0	7.2	1	6.9	0	7.1
2	6.4	0	7.0	1	6.6	1	6.8	0	7.0	1	6.2	0	7.2	0	7.2	1	7.0	0	7.2
4	4.2	0	7.0	1	6.8	1	6.8	1	6.9	0	7.2	0	7.0	0	7.1	0	7.2	0	7.2
2	5.8	0	7.0	4	6.2	2	6.6	2	6.0	0	7.2	0	7.0	1	6.8	2	6.9	3	6.4
2	5.8	0	7.2	4	6.0	0	7.1	2	6.2	0	7.0	0	7.1	1	6.8	2	6.9	3	6.4
4	5.0	0	7.2	1	6.8	1	6.6	1	7.0	1	7.0	0	7.1	1	7.0	1	7.0	0	7.0

been stated, the rise of the vaseline cap in the tube is an excellent indicator of the quantitative production of gas. The size of the test tube, the amount of medium used, amount of vaseline used and concentration of the sugar all being constant factors, it is reasonable to suppose that this method of measurement is accurate.

In studying anaerobic bacteria, egg albumin is ordinarily used as an indicator of acid production. In these tests, however, the actual hydrogen-ion concentration of each culture was taken after the method of Clark and Lubs.[16]

The results of the sugar fermenting properties of these are reported in Table I.

As will be seen later when the proteolytic reactions are taken into consideration, it is quite feasible to divide certain of the anaerobes into strongly proteolytic, feebly proteolytic and non-proteolytic (or saccharolytic) groups, respectively. However, from this it should not be inferred that certain members of the proteolytic group do not as well have broad sugar splitting properties. A brief consideration of the following findings shows this clearly.

B. oedematis maligni, one of the most powerful of proteolyzers, splits glucose, levulose, galactose, saccharose, raffinose and mannose with an appreciable amount of gas and acid production. It seemed to only feebly attack maltose and lactose, judging by the production of gas, but when measurement was made of the hydrogen-ion concentration, fairly marked reduction was found to have occurred in these last named sugars.

B. tetani copiously produced acid and gas with glucose, levulose and galactose, while with saccharose, maltose, lactose, raffinose, mannose and xylose, much acid formation took place with only a few bubbles of gas.

B. histolyticus gave no sign of gas production with any of the sugars, and lack of this property forms a point of differential importance. Acid production was noted in the presence of maltose, lactose, xylose and inulin.

Henry noted a fermentative action for this organism with glucose and levulose, but states his suspicions as regards the

purity of his culture. Weinberg and Seguin [17] observed B. histolyticus to be the only anaerobic bacterium capable of producing a non-putrifying type of proteolysis *without* infiltration of gas in tissue.

B. sporogenes, another frankly proteolytic organism, readily split glucose, levulose, galactose, saccharose, maltose, lactose, and raffinose. Henry only records positive reaction with glucose, levulose and saccharose. Weinberg and Seguin pronounce B. sporogenes to be the only anaerobic organism capable of *alone* producing proteolysis and gas infiltration in tissue.

B. botulinus according to my observations attacks only the monosaccharids thus agreeing with observations made by most other investigators of this group.

B. bifermentans produced acid and gas with glucose, levulose and maltose but acid alone with galactose, saccharose, lactose, raffinose and xylose.

B. bellonensis seemed to be capable of splitting glucose, maltose, and, to a small extent, raffinose and xylose, that is, as concerns both acid and gas production. However, acid alone was produced with levulose, galactose, saccharose, lactose, glycerine and arabinose.

B. oedematiens, initially observed by Weinberg [18] as powerful in splitting carbohydrates, in the hands of Henry only altered glucose, levulose, maltose, xylose and starch. I have obtained positive reactions with glucose, levulose, galactose, saccharose, maltose, lactose, raffinose, mannose, xylose, and starch, with acid production on inulin; B. oedematiens is one of the most fastidious organisms as regards its anaerobic and nutrient requirements. The cap of paraffin oil as used by Henry seems here especially insufficient to produce the necessary anaerobiosis. The medium used by him, composed of one part casein digest fluid and two parts physiological salt solution, gave rise to but scanty growth. This may account for our rather wide divergence in regard to the sugar splitting properties of this organism.

B. aerofoetidis is peculiar in giving a constant negative saccharose reaction.

Vibrion septique, and the bacillus of Ghon and Sachs, act

upon the carbohydrates in an identical manner. For this reason and on account of their other cultural similarities, I am inclined to support Heller's opinion concerning their probable identity.

B. welchii. Two strains of this organism proved to be members of group four, while two other cultures were subsequently placed in group two, agreeing with Henry and with Simonds'[19] original classification, with the exception that the presence of an enzyme capable of producing acid and gas upon xylose was noted. It is probable that the superior anaerobiosis afforded by the use of the vaseline cap is responsible for bringing to light the action of this xylose-splitting ferment.

B. fallax. With the exception of B. fallax, all of the carbohydrate reactions were completed within forty-eight hours. B. fallax invariably lagged behind and on the majority of sugars evidence of activity only began at the end of that time. For this reason, the B. fallax readings were taken only after seventy-two hours. This seems to be a point of considerable diagnostic importance. A lactose fermenting enzyme, unobserved by Henry, but noted by Weinberg, was present in the culture studied.

B. tertius is most commonly confused with B. welchii and the sugar fermenting properties displayed by the former offer a means of differentiation of singular value, in that gas is produced in about half as great volume as in the case of the latter and the reduction in the hydrogen ion concentration is not nearly so marked. B. tertius seems capable of utilizing nearly all of the sugars tested. The strain in my collection had an additional property of fermenting inulin, with acid and gas production. This faculty, I believe, has not been observed.

It seems that the sugar fermenting reactions of the anaerobes offers a desirable means of differentiation, and brings to light minute differences which may be present. Observers have recently advocated the classifying of B. sporogenes, B. oedematis maligni and B. putrificus as identical organisms. While it is true that the proteolytic activities of these organisms are in some respects the same, their varying action upon sugars makes one doubtful of the desirability of such a grouping. As

will be seen, organisms such as Vibrion septique, B. oedematiens and B. tertius are similar in action upon the so-called proteolytic types of media but one would hardly advocate placing these under one head, upon such slight cultural evidence. However, on account of the marked similarity of Vibrion septique and the bacillus of Ghon and Sachs as regards their saccharolytic and proteolytic activities, I find myself in agreement with Heller's suggestion to the effect that these organisms are identical.

PROTEOLYTIC TESTS

Anaerobic spore-bearing bacteria have heretofore been divided into proteolytic and saccharolytic groups. Previous observers have found that certain members of the saccharolytic group show, in old cultures, some proteolysis, which has been assumed to be due to the development of secondary proteolytic ferments. In our studies no such evidence of secondary proteolysis ever occurred in cultures of B. welchii, B. oedematiens, B. tertius, B. fallax, Vibrion septique, and the bacillus of Ghon and Sachs, although observations were made over a period of eight months and the organisms were grown on media suitable for evidence of proteolysis. The organisms included in this group should therefore be described as *non-proteolytic*.

Since the cultures with which we worked were known to be pure it seems probable to us that the late proteolysis described by other observers may have been due to contaminating organisms.

The media used, together with their formulae, are given here. Care has been taken to select substances which would give well defined reactions.

Results have been tabulated, not under the heading of organisms but under that of media. In other words, the reaction of the collection has been described upon each medium as a heading, rather than the reaction of the organism to each individual substance. In this way points of differential importance stand out more clearly.

The methods used for inoculating and obtaining anaerobiosis are essentially the same as have been described. Due to the

nature of the media used, especial diligence was exercised to prevent contamination. The media just prior to use were subjected to twenty-four hours incubation as a precaution against the use of contaminated material. All had been sterilized in the autoclave for twenty minutes at fifteen pounds pressure.

The cultures comprising the tests were kept in the incubator for forty-five days, and almost daily observations were made upon them. They were then allowed to stand at room temperature, and examinations were made from time to time. At the end of eight months a final reading was taken, together with the hydrogen ion determination of each individual culture.

Observations have been given in detail as the writer agrees with the criticism that this has not been done in the previous descriptions of cultural reactions of these organisms.

Media used. Seven types of media were used. 1. Cooked meat medium; 2. Coagulated egg albumin cube broth; 3. Alkaline egg fluid (also modified); 4. Brom cresol purple milk; 5. Cooked cod fish medium; 6. Gelatin; 7. Inspissated serum.

Cooked meat medium (Robertson).[9] Reactions upon this substance are stated by Robertson to be comparable to the results obtained upon the "Hirnbrei" or brain medium of von Hibler.[36] The formula for cooked meat medium is widely known and no repetition of it will therefore be given here. Instead of determining the reaction with litmus paper as advocated by Robertson the writer adjusted the substance to Ph. 7.2.

Coagulated egg albumin broth. A few eggs are boiled for fifteen minutes to insure complete coagulation of the albumin. The white is then carefully separated from the yolk and washed in distilled water to completely free it from the yellow substance. The albuminous portion is then cut into cubes of about 2 cm. and one of the cubes is placed in each of several test tubes. The tubes are then filled with 10 c.c. of broth made by the usual formula, namely, beef-infusion broth plus 2 per cent casein digest fluid — reaction Ph. 7.2 — and autoclaved for twenty minutes at fifteen pounds pressure.

This medium besides being simple to prepare is a valuable one because of the variation of action occurring upon it. Some points of differential importance are here brought to light which are not in evidence in tests upon other substances.

Cooked cod fish medium. Prepared from fresh cod fish steaks as follows:

The fish is freed from bone and skin, chopped into fine particles and further reduced by a short period of grinding in a mortar. 800 c.c. of tap

water are added for each pound of fish and the whole allowed to boil
gently for forty-five minutes. Adjust the reaction to Ph. 7.2. Just
prior to testing care must be taken to thoroughly mix the fish muscle and
broth so that one verticle inch of the tissue may be present in each tube.
The substance is autoclaved for the usual length of time.

Inspissated Ox Serum. Prepared from Difco powder. The medium
was brought to a hydrogen ion concentration of 7.2 and inspissated verti-
cally in the tubes. The proteolytic action upon coagulated serum was
therefore not that of a surface growth but actually a deep stab culture.
One must exercise especial care in inspissating to preclude the formation
of air bubbles, as these often give a false impression concerning gas
formation.

Milk — Brom Cresol Purple. Partially skimmed, adjusted to Ph. 7.2,
tubed in 10 c.c. amounts and autoclaved. Prior to tubing, 40 c.c. of
Brom credol purple indicator was added to each liter of milk.

Gelatin. The gelatin was made a little stiffer than is usual. Twenty
per cent gelatin was used, made up in beef-infusion broth. No casein
digest substance or glucose was added. The medium was as usual ad-
justed to Ph. 7.2 and autoclaved.

Alkaline Egg Fluid, Robertson.[9] As Miss Robertson states this is a
modification of Besredka's substance.
The yolk of one egg and the whites of two are beaten up in a beaker
and to this mixture is added 6 c.c. of N/1 NaOH. Five hundred c.c. of
tap water are then added gradually. Heat very slowly to 95° C. keeping
the mixture at this temperature for about an hour. Filter through cotton
wool and muslin, tube and autoclave. When the hydrogen ion concen-
tration of this medium was taken it was found to be 8.2. As has already
been pointed out, this was in my hands far above the optimum. Never-
theless a series of tests was performed upon medium with this reaction
for its comparable value. In a second series the writer found it advisable
to adjust the reaction to Ph. 7.2. When this is done, some of the albumin
does not go into solution but remains at the bottom of the tube in the
form of a white precipitate. The modified substance has the appearance
of a milky emulsion, while as made by Robertson it is golden yellow in
color. In Robertson's medium several of the organisms refused to grow
at all. Upon the more acid substance, however, each organism grew
regularly giving rise to changes more rapidly and of a more distinctive
nature than was noted upon the more alkaline fluid.

The necessity for having the media properly adjusted as re-
gards the hydrogen ion concentration cannot be too strongly
emphasized. These anaerobic spore bearing organisms are
just as sensitive to proper environment as are any of the other
types of bacteria and to obtain maximum growth and biologi-
cal action their requirements must be carefully ascertained and

provided for. An excess of alkalinity in the media seems to have been provided by most investigators of this group, probably on the assumption that the bacteria themselves produce a marked degree of alkalinity. This, as will presently be shown, is much lower than has heretofore been supposed.

REACTION UPON COOKED MEAT MEDIA

B. sporogenes. After the first twenty-four hours there is rather a marked production of gas amounting to a rise of 1 cm. of the vaseline cap. The meat is much darkened and distinctive evidence of proteolysis is to be noted. White flaky crystals of what afterwards proved to be tyrosine are produced and the broth is heavily clouded. Upon puncturing the vaseline cap one is struck by the penetrating odor of putrefaction. After forty-eight hours the gas production is usually doubled and in some cases tripled. The meat is entirely darkened and the crystals of tyrosine seem to be about twice as profuse. Gas slightly increases as proteolysis progresses and on the eighth day the meat will be seen to have been reduced fully one-half in volume. After this point there is no further production of gas but digestion and crystal production continue more or less actively. At the end of three weeks the fluid portion of the culture is still cloudy. There is profuse crystal production giving the remaining small amount of red-black meat residue a peculiar mottled appearance. At the end of a month the meat is about three-fourths reduced in volume and the broth is still for the most part turbid, there being however a slight tendency toward clearing at the top.

The broth stays turbid for about three months, clearing only very gradually. Eight months after planting the muscle tissue is practically all digested, leaving only a small amount of reddish-black residue streaked with white tyrosine crystals.

The reaction of B. sporogenes upon this medium is characterized by its profuse gas production, its profuse production of tyrosine crystals even after forty-eight hours, its marked darkening of the meat with subsequent rapid digestion, the production of a powerful and disagreeable odor of putrefaction, and persistent clouding of the broth.

The reaction of this culture at the end of eight months reads Ph. 7.6.

B. botulinus. After twenty-four hours incubation gas is produced to the extent of about 1 cm. rise of the vaseline cap. The meat particles are nearly all blackened and the fluid is turbid. The odor of putrefaction is milder than that of B. sporogenes and not nearly so disagreeable. When forty-eight hours have elapsed the volume of gas produced is about doubled, the meat is totally darkened with swelling of the particles. Four days after seeding the meat has diminished about one-quarter in volume.

Usually on the tenth day the fluid starts to clear, increasing the sedimentation daily until about twenty days in all have elapsed, after which the fluid may be called clear. There is gradual increase in gas production

as digestion proceeds until the vaseline cap rises to about 3 cm. The meat on the twentieth day is only about one-half as great in volume as at the start. There is little change from the above over a period of eight months, at the end of which time the meat is about two-thirds diminished in volume, leaving a dirty reddish black residue streaked with a small number of tyrosine crystals. On the whole the reaction just described resembles that of B. sporogenes except that the production of tyrosine is not nearly so marked and the meat is not so greatly digested. Gas is not evolved in as large quantities, the odor of putrefaction is not as foul and disagreeable, and the fluid is not nearly as tenaciously turbid as that of B. sporogenes, clearing as it does at the end of twenty days.

The reaction of the culture of B. botulinus upon this medium at the end of eight months was Ph. 7.6.

B. putrificus. Twenty-four hours incubation upon this medium marks the production of gas equal to about one-half cm. rise of the vaseline cap. There is some black decolorization of the meat in the extreme bottom of the tube and a penetrating foul odor similar to that produced by B. sporogenes. After forty-eight hours the volume of gas is about doubled and the zone of darkening of the meat increases somewhat in its upward trend. Proteolysis is not at this time in evidence. No further alteration occurs until after the fourth day when dark pigmentation is produced in about two-thirds of the meat. The tenth day finds the meat entirely darkened, gas production increased to about three cm., and digestion equaling about one-fourth of the muscle tissue. The broth of this culture remains tenaciously cloudy, beginning to clear only after about twenty days have elapsed. This process continues gradually until about forty-five days after incubation when the broth will be found to be perfectly clear.

Eight months after planting the meat is probably about one-half digested, leaving about an equal portion of dark red residue. Only a very few crystals of tyrosine are produced. The reaction of this culture at the end of eight months reads Ph. 7.4.

B. putrificus acts much more slowly upon this medium than does B. sporogenes. Although there are some points of similarity the rapidity with which B. sporogenes proteolyzes the muscle tissue forms a distinctive point of differential value. This observation agrees with those made by Hall [21] and others, who found B. putrificus to be rather tardy in its proteolytic activity.

B. tetani. This organism produces about 3 cm. of gas after the first day of incubation. The meat assumes a slate gray color and the broth is profusely clouded. After four days gas production is slightly increased and feeble proteolysis is apparent in the top layers of the meat. Digestion gradually progresses until the fourteenth day when the muscle tissue will be found to have been reduced about one-fourth of its volume, and at this time the slate gray color of the meat changes to a somewhat darker color, but not nearly so dark as the color produced by other organisms more frankly proteolytic in their nature. At this time, also, the erstwhile

cloudy fluid will be found to have cleared materially. Forty-five days after planting the meat will have decreased about one-third of its total volume. Even after eight months have elapsed there is no further change. The meat residue is well streaked with crystals which at this time are not believed to be tyrosine. They do not exhibit the characteristic needle-like wheat sheaf appearance but are more chalky in aspect. Identification of this substance is at present under investigation. The reaction of this culture at the end of eight months was Ph. 7.2. B. tetani gives rise to a foul odor but it is not nearly as markedly so as that produced by B. sporogenes. Gas production is more rapid and profuse and the culture is more gradually proteolytic, resembling in the latter respect B. putrificus more than does any one of the other organisms but producing much more gas.

B. oedematis maligni. The reaction of this organism upon cooked meat medium strongly resembles that of B. sporogenes. In fact it may be said that there are really no points of differential value to be seen as regards this medium.

In the first twenty-four hours we have production of gas in amount almost identical with that by B. sporogenes, the meat is darkened with distinctive evidence of proteolysis, and a few crystals of tyrosine are found. After forty-eight hours gas production is more than doubled and the meat assumes the dark red-black hue. Proteolysis continues rapidly, the broth remains tenaciously turbid, clearing only after three months have passed. (In fact some slight evidence of clouding was even noted after eight months.) At the end of eight months the meat as in the case of B. sporogenes will be found to be entirely proteolyzed, there being only the dirty red-black detritus copiously streaked with tyrosine crystals. The odor characterizing this reaction is penetratingly foul and disagreeable. Reaction after eight months was Ph. 7.6.

B. histolyticus. The growth of B. histolyticus upon this medium is quite characteristic for here we have progressive proteolysis continuing at rather a rapid rate with practically no gas production. When gas production does occur, it is so feeble that it is marked, at the most, by the occurrence of only three or four small bubbles under the vaseline cap.

A day after planting the meat turns a dirty chocolate brown and there is already evidence of digestion in the top layers. After forty-eight hours the color deepens and digestion extends a little further, embracing about one-quarter of the total volume of the muscle tissue. Usually after about six days the characteristic balls of tyrosine begin to appear, and indeed this formation seems to be a characteristic of B. histolyticus alone. In the case of all other organisms producing tyrosine the crystals occurred in the usual flaky needle-like form.

On the fourteenth day the very cloudy fluid begins clearing, and at this time the meat is about one-half reduced in volume, the residue assumes a deeper red-black hue. Balls of tyrosine are at this time very numerous. Digestion increases gradually and after thirty days only about one-third of the meat remains.

After eight months the meat is practically all digested, leaving a small amount of black residue. The balls of tyrosine still retain the spherical form. The fluid will be still found to be somewhat cloudy. The odor of this culture while putrefactive is very mildly so. The reaction is by far the most alkaline encountered, being at the end of eight months Ph. 8.4, probably due to the fact that digestion is carried to the ammonia stage.

Upon this medium the action of B. histolyticus is characterized by lack of gas production, its gradual but complete proteolysis, the marked production of alkaline substances, probably ammonia, and the occurrence in spherical shape of tyrosine crystals.

B. bifermentans. This organism seems rather intermediate between the frankly proteolytic and non-proteolytic or saccharolytic groups.

Its reaction upon cooked meat medium during the first twenty-four hours leads one to believe that it will be subsequently classified as a representative of the proteolytic group, as the meat becomes markedly darkened and there is some gas production (about 1 cm. rise of the vaseline cap), most of the active proteolyzers beginning their reaction in just this way. However the above conditions remain unaltered for twenty days or more, there being no further evidence of proteolysis or gas production, and the only change noted at that time is that the erstwhile cloudy broth which has been gradually clearing has now become perfectly clear. After forty-five days in the incubator, feeble proteolysis is noted in the extreme top layers of the meat. Eight months after seeding the meat is about one-fourth digested, leaving the otherwise unaltered muscular tissue much blackened: the odor is only faintly putrefactive. The reaction of the culture is at this time Ph. 6.8. There is no production of tyrosine or other crystals.

B. bellonensis. From its action upon cooked meat medium I am inclined to regard this organism as a member of the so-called intermediate group of spore-bearing anaerobes. The very feeble proteolysis is not even as marked as in the case of B. bifermentans.

After twenty-four hours have elapsed the meat is very slightly darkened in the extreme depths of the culture. No gas is at this time produced. After four days there is a small amount of gas produced amounting to 2 cm. or less — and the slightly clouded fluid at this time becomes clear. No proteolysis is to be noted. There is little change in the culture from this time on. The zone of darkening extends until about one-half of the meat is so affected and at the end of five months there is a trace of digestion in the extreme bottom of the meat.

Reaction after eight months Ph. 6.8.

B. aerofoetidis. About one cm. of gas is produced at the end of twenty-four hours incubation: otherwise there is no change. After forty-eight hours the meat in the lower half of the tube becomes much darker in color, but is not blackened as in the case of the frankly proteolytic organisms, or B. bifermentans or B. bellonensis. There is no change from this time on until the eighth day when the fluid becomes clear. Gas production is not increased. There is but little further alteration.

After eight months slight traces of proteolysis are to be noted in the extreme bottom of the meat. The odor of the culture is not penetratingly foul but slightly foetid, one might say in an acid way.

Reaction eight months after planting, Ph. 7.00.

B. oedematiens. Copious gas production marks the first twenty-four hours incubation, often occurring in amounts as great as 3 cm., the average being about 2 cm. The meat is markedly reddened in color. On the fourth day' or thereabouts, the fluid becomes clear. This is in striking contrast to the clearing of the broth in the case of the proteolytic organisms, which, as has already been pointed out, usually takes much longer and in some cultures is not complete even after eight months.

The meat gradually becomes a lighter red until after about twenty days incubation it assumes a salmon pink hue which is permanent.

After eight months the culture remains practically unaltered. The particles of muscular tissue may be said to be somewhat reduced in individual size but there is no reduction in the total volume of the meat. Reaction at the end of eight months, Ph. 6.6. The gas produced by B. oedematiens upon cooked meat medium is not as great in amount as that produced by B. welchii but is of larger volume than that produced by Vibrion septique.

Vibrion septique. The reaction of this organism upon our cooked meat medium agrees with the observations made by Heller [10] to the effect that the organism is not in the least proteolytic and gives a typical non-proteolytic or saccharolytic reaction.

After twenty-four hours incubation usually about 2 cm. of gas are produced the meat becomes a bright copper red. There is no change until the eighth day when the fluid clears.

The above described situation remains constant. There is no further production of gas. After eight months the meat particles slightly diminish in size but there is absolutely no reduction of the total volume of tissue. Reaction after eight months, Ph. 6.4.

B. of Ghon and Sachs. As for Vibrion septique.

B. fallax. In my hands B. fallax showed no evidence of growth upon this medium at the end of twenty-four hours. After forty-eight had elapsed, however, sufficient gas was produced to account for 1 cm. rise of the vaseline cap, the meat tending to become red. On the third day the meat particles assume a bright pink color which does not fade or become altered even after eight months. After eight days of incubation the broth becomes clear. There is no deviation from the above. No black pigment was noticed as reported by Henry.[14] There is absolutely no evidence of proteolysis. Reaction after eight months, Ph. 6.5.

B. welchii. The action of B. welchii upon this medium is clearly defined and it is relatively an easy matter to differentiate it from other organisms of the non-proteolytic group.

After twenty-four hours there are produced between 4 and 5 cm. of gas; the meat particles become somewhat swollen and very red. Gas is produced with such violence as to suggest that the culture is receiving gentle boiling. B. welchii doubtlessly produces more gas on this medium than does any other organism and unless sufficient vaseline is applied to the top of the culture the cotton plug, with the vaseline cap, will invariably be blown from the tube. At the end of forty-eight hours the total amount of gas is somewhat increased but the violence of the reaction has markedly subsided. This remains unaltered until the fourth ·day, when the slightly clouded fluid becomes clear. After ten days the particles of meat may be said to be somewhat reduced in size but there is no reduction in the total volume of the tissue. There seems to be but little further alteration. At the end of eight months the meat is still brilliantly red, appearing almost carmine in color. Even after a lapse of eight months there was not the slightest evidence of proteolysis or black pigment production.

B. welchii is identified with ease upon cooked meat media. It produces more gas than does any other member of the saccharolytic group. The meat particles are thus more enlarged and the meat takes on a brighter hue of red than is seen in the case of any of the others. Reaction after eight months, Ph. 6.6.

B. tertius. The action of this organism upon cooked meat media somewhat resembles that of B. welchii. However, during the first twenty-four hours B. tertius does not produce nearly as much gas and the color assumed by the meat is not as deep a red, tending more toward a salmon pink. The fermentation subsides at the end of forty-eight hours. There is no further change until the twentieth day when the fluid becomes clear. (It will be noted that the fluid portion of the culture of B. welchii clears at the end of four days.) After thirty days of incubation the meat assumes a light salmon pink color in marked contrast to the permanent deep red of B. welchii.

Eight months after planting, the culture shows no further alteration. There is no proteolysis nor production of black pigment. Reaction after eight months, Ph. 6.8.

Brom Cresol Purple Milk

B. sporogenes. Gas is produced in this medium after seventy-two hours of incubation and on the fourth day there is clotting of the milk. On the sixth day the clot, still retaining its cylindrical shape, separates from the walls of the tube and is surrounded by a small amount of turbid fluid. Digestion proceeds rapidly and after eight days nothing remains of the clot save a very small cylindrical fragment. A large amount of gas is at this time in evidence, amounting to a 3 cm. rise of the vaseline seal. The milk is replaced by turbid yellow fluid. There is no alteration of the above condition for some months. The fluid clears very gradually, and after six months have elapsed, the small fragment of clot entirely disappears. After eight months the hydrogen ion concentration of the clear, straw colored liquid reads Ph. 6.8.

B. botulinus. On the third day of incubation the milk is firmly clotted but no production of gas is at this time in evidence. On the fourth day the clot is fully two-thirds digested, leaving a small amount of flaky, mushy, unorganized material in the bottom of the tube. After eight days the fluid will be found to have cleared, while the flaky residue at the bottom of the tube is markedly reduced in volume. There is but little change from the above for some months. After eight months, the flaky residue is practically all digested, leaving only a few, small white fragments. Hydrogen ion concentration of the clear straw colored fluid was Ph. 6.8. There is no gas produced throughout the entire reaction.

B. putrificus. There is feeble production of gas in the milk culture after four days of incubation and, when ten days have elapsed, the milk which has become clotted is reduced about one-quarter in volume. It is overlayed with cloudy fluid. After fifteen days of incubation the mushy, flaky remaining clot is reduced about one-half of its total volume, and at this time also, the fluid clears, leaving a light straw colored liquid. There is no change from the above. After eight months the hydrogen ion concentration of the liquid was Ph. 6.6.

B. tetani. This organism attacks the milk but slightly. Feeble production of gas marks the third day of incubation, accompanied by the production of a hard acid clot. This is reduced about one-quarter of its volume after ten days of incubation, at which time also, it will be found to have contracted and shrunken away from the walls of the tube, being displaced by clear, watery fluid. There is no further alteration. The hydrogen ion concentration of the culture after eight months reads Ph. 6.4.

B. oedematis maligni. After twenty-four hours of incubation, gas is produced sufficient to account for a 1 cm. rise of the vaseline cap. On the sixth day the milk forms into a soft fragile clot, and from this time on digestion proceeds rapidly. After eight days the clot is digested to about one-half of its volume, being overlayed by clear watery fluid. After twelve days nothing remains of the clot save a small cylindrical fragment, which entirely disappears at the end of thirty days. Gas production is not increased from the amount mentioned above. Hydrogen ion concentration after eight months, Ph. 6.8.

B. histolyticus. B. histolyticus does not alter the milk until after the third day of incubation, after which time a soft mushy clot is produced. No gas is formed at this time nor at any time throughout the reaction. On the sixth day the mushy substance is about one-half digested, being overtopped with clear fluid. On the eighth day the clot is probably two-thirds reduced and ten days later it is entirely digested. A clear, light, straw colored fluid replaces it. There is no further change. Hydrogen ion concentration after eight months, Ph. 7.00.

B. bifermentans. In the milk culture of this organism, there is clotting after the second day of incubation. The clot is soft and granular. After

the fourth day it is reduced in volume about twenty-five per cent, and five days later it is at least three-quarters digested. The fluid clears on the tenth day. No gas is produced at any time throughout the reaction. B. bifermentans shows more evidence of proteolysis upon the milk medium than in any other substance. There is no further change from the above. The hydrogen ion concentration of the clear, straw colored liquid reads after eight months, Ph. 6.5.

B. bellonensis. The action upon milk is similar in all respects to that produced by B. bifermentans, with the exception that instead of producing a soft granular clot, that produced by B. bellonensis is quite hard and firm. Hydrogen ion concentration after eight months, Ph. 6.6.

B. aerofoetidis. The milk is formed into a hard acid clot after twenty-four hours incubation. No gas is produced. There is little alteration of the above over a period of months. At the end of eight months the clot will be found to have shrunken to some extent and to have squeezed away from the walls of the tube. There is at this time a trace of gas. Hydrogen ion concentration, Ph. 6.2.

B. oedematiens. Gas is produced by this organism at the end of forty-eight hours incubation. Clotting, however, is not in evidence until after the fourth day. The solid acid clot slightly separates from the vaseline seal, leaving a ring of clear liquid, at the top. There is no further alteration. After eight months the clot will be found to have shrunken away from the walls of the tube, not to a marked degree, however. Hydrogen ion concentration, Ph. 5.6.

Vibrion septique. Vibrion septique does not alter the milk until after six days of incubation. A firm acid clot is then produced, and sufficient gas to account for the vaseline cap rising about 1 cm. After fourteen days the firm clot becomes somewhat split, and there is separation at the top and sides of the tube. The separated portion being replaced by clear liquid. This marks the end point of the reaction. After eight months the hydrogen ion concentration of the culture is Ph. 5.8.

B. of Ghon and Sachs. The reaction upon milk is identical with that described for Vibrion septique.

B. fallax. B. fallax gave no evidence of activity upon the milk until after twenty days of incubation. It then clotted the milk into a very loosely organized mass, with the production of a few bubbles of gas. There was no further alteration. Hydrogen ion concentration after eight months, Ph. 6.6.

B. welchii. The characteristic stormy type of action was invariably produced by the cultures of B. welchii. It is so well known that no further description of it will be given here. Hydrogen ion concentration after eight months, Ph. 5.2.

B. tertius. In contradistinction to the action of B. welchii, B. tertius produced a soft clot in the milk after forty-eight hours incubation, accompanied by a few bubbles of gas. There was no further evidence of activity until after a number of months, when slight contraction of the clot was to be noted, leaving a small amount of clear fluid at the top and sides of the tube. Hydrogen ion concentration after eight months, Ph. 5.9.

SOLIDIFIED EGG ALBUMIN CUBE BROTH

B. sporogenes. Twenty-four hours: The cube of egg solidified albumin is in this time about one-half digested, about ½ cm. of gas is produced, profuse clouding of the broth takes place, and there is an extremely foul odor.

Forty-eight hours: Cube of egg albumin about three-quarters digested, leaving a small brown fragment. Gas increased to about 1 cm. rise of the vaseline cap in the tube.

Seventy-two hours: Egg albumin is at this time entirely proteolyzed, leaving a small amount of residue composed of a white slimy substance. Gas slightly increased to about 1½ cm.

There is no further change through forty-five days of incubation. The broth does not begin to clear until about the sixtieth day, gradually clearing from then on, until at the end of about ninety days there is no further evidence of clouding. Observation for eight months gives no further information. At no time is there any black pigment produced in the fluid nor is there any blackening of the egg albumin cube. The reaction seems characterized by its rapidity of digestion, foul odor, production of gas, and tenacious clouding of the broth.

B. botulinus. Twenty-four hours: About 1 cm. of gas is produced and the cube of egg albumin is about one-half digested. The fluid is heavily clouded.

Forty-eight hours: Gas production is at this time increased to about 2 cm. rise of the vaseline cap and the cube of egg albumin is at least two-thirds digested.

Seventy-two hours: The small remaining undigested fragment of the cube is at this time rendered rather translucent. Gas is not increased.

The unproteolyzed fragment of albumin decreases in size daily and takes on a dirty brown color. After eight days nothing remains save a tiny brown translucent fragment. There is no further change until the fourteenth day when the fluid will be found to have begun to clear. At the end of twenty days the albumin is entirely digested, leaving only a trace of unorganized residue. The fluid at this time is only faintly clouded. This reaction remains unaltered until after about sixty days, when the broth has become clear. There is no further action. Reaction at the end of eight months, Ph. 7.4. As will be noted, the digestion is more gradual than that carried on by B. sporogenes.

B. putrificus. Twenty-four hours: Gas production enough to cause 1 cm. rise of the vaseline cap; also digestion of the egg albumin amounting to about one-quarter of its volume. After forty-eight hours the remaining fragment becomes translucent. This fragment diminishes in size daily and on about the eighth day it will be found to have entirely disappeared, leaving a small amount of unorganized mucoid deposit in the bottom of the tube. This reaction remains unchanged. At the end of forty-five days the heavily clouded fluid has finally become clear. Hydrogen ion concentration after eight months, Ph. 7.4.

It is quite evident that these proteolytic organisms do not alkalinize the media to as great an extent as has been heretofore supposed.

B. oedematis maligni. The action of B. oedematis upon this media is characterized by its rapidity. At the end of twenty-four hours the cube of egg albumin is nearly all digested, only a small fragment remaining. There is slight gas production amounting to about 1 cm. rise of the vaseline cap in the tube. After forty-eight hours, gas production is somewhat increased. The particle of undigested egg white is at this time reduced in size to a tiny dark brown speck. On the third day of incubation it turns black and after four days have elapsed, entirely disappears. The heavily clouded broth begins to clear at the end of twenty days, gradual precipitation occurring until after about the sixtieth day it will be found to have entirely sedimented. Reaction after eight months, Ph. 7.4.

B. tetani. This organism gives rather a characteristic reaction upon the egg albumin cube, several duplicate cultures, which were planted, invariably producing the same peculiar type of decomposition.

At the end of twenty-four hours gas is produced to the amount of about 2 cm. rise of the vaseline cap in the tube. The cube of egg albumin is about one-fourth reduced in size but, strange to say, the disintegrated substance remains as a slimy, stringy halo about the unattacked portion of the cube. The amount of gas produced is doubled after forty-eight hours and the peculiar process of decomposition at this time embraces about one-half of the cube. As usual, the reaction is accompanied by profuse clouding of the broth. Slow proteolysis of the type described proceeds. On the eighth day the fluid gradually begins to clear and about a week later completes its process of sedimentation. On the forty-fifth day the cube of albumin is practically two-thirds digested, the attacked portion appearing not to go into solution but remaining about the unaltered cube in the peculiar halo-like manner. At the end of eight months there is no further evidence of digestion, nor is the above description at all altered. Reaction at this time, Ph. 7.00.

B. histolyticus. No gas is produced on this medium at any time throughout the reaction.

When twenty-four hours have elapsed the broth becomes a little clouded and a slight chipping off of fragments of the albumin cube is to be noted. This unusual type of digestion proceeds slowly, small fragments being chipped off from time to time. Some of these go into solution while others remain at the bottom of the tube in an unaltered condition.

This peculiar process of digestion gives the cube a curious appearance of irregularity especially about its margins. The fluid is clear at the end of thirty days. After forty-five days of incubation the cube may be said to be reduced about one-half in volume. There are at this time numerous fragments of unattacked albumin at the bottom of the tube. No further change is noted during the eight months of observation. The reaction at this time is Ph. 7.7.

B. bifermentans. The reaction about to be described seems characteristic for the above-named organism. That it is not a chance occurrence has been determined by repeating the test on several occasions.

At the end of twenty-four hours the fluid is noticeably clouded; the egg albumin cube, however, seems unaltered. No gas is at this time produced and after forty-eight hours the reaction is complete with the exception of a gradual clearing of the fluid. It is to be noted that the edges of the cube have been very noticeably attacked. They appear irregularly scalloped, giving the impression of having had small fragments removed at intervals. The edges are also translucent for some little distance towards the center. However, that which is of greatest interest is the stringy mucoid type of digestion which takes place at the bottom of the cube. This slimy, elastic product forms a sort of cushion on which the unattacked portion of the egg albumin rests. When the tube is inverted one end of the elastic material remains attached to the bottom of the tube while the other end clings fast to the cube, which is at this time in contact with the vaseline cap, thus stretching a wide white ribbon of altered egg albumin from top to bottom of the tube. One cm. rise of the vaseline cap is indicative of the production of that amount of gas. There seems to be no reduction of the total volume of the albumin. The broth gradually clears in about thirty days. Even after eight months there is no further alteration. Hydrogen ion concentration, Ph. 6.8.

B. bellonensis. After twenty-four hours incubation there is marked clouding of the broth, together with enough gas production to raise the vaseline cap about 1 cm. At the end of forty-eight hours slight evidence of proteolysis is to be noted around the lower portion of the cube. The fluid clears in about forty-five days. During eight months of observation there was no further change. Ph. 7.00.

B. aerofoetidis. Extremely feeble proteolytic action is the cultural characteristic of B. aerofoetidis upon this medium.

Aside from profuse clouding of the broth there is no reaction until the third day when enough gas is produced to cause a 2 cm. rise of the vaseline cap. On the eighth day one notices that the edges of the albumin cube are very slightly attacked. The tenth day of incubation marks a slight clearing of the broth. This continues gradually until the forty-fifth day, when the fluid becomes clear. There is no further proteolysis. Hydrogen ion concentration after eight months, Ph. 6.6.

B. oedimatiens. Gas enough to cause the vaseline cover to rise 1 cm. is produced at the end of twenty-four hours. There is no apparent

proteolysis and no further gas production. The fluid begins the process of clearing on the third day and after ten days there is no further evidence of clouding. Even after eight months there is no hint of digestion. Hydrogen ion concentration, Ph. 6.6.

Vibrion septique. After twenty-four hours incubation there is enough gas produced to raise the vaseline about 0.5 cm. The fluid is cloudy but not nearly so much so as in the case of the other organisms studied upon this medium. The fluid begins to clear on the eighth day and completes the process on the twentieth day. There is no suggestion of proteolysis. Hydrogen ion concentration after eight months, Ph. 6.6.

B. of Ghon and Sachs. Identical with Vibrion septique.

B. fallax. Aside from a slight clouding of the broth which clears at the end of four days, and a few small bubbles of gas, there is no action upon this medium. At no time is there even the slightest evidence of proteolysis. Hydrogen ion concentration after eight months, Ph. 6.9.

B. welchii. Twenty-four hours at incubation temperature marks the production of gas sufficient to raise the vaseline cap 1½ cm. This doubles after forty-eight hours. There is profuse clouding of the broth which clears after about four days. There is no alteration in the cube of egg albumin, even after eight months. Hydrogen ion concentration after eight months, Ph. 6.4.

B. tertius. After the first twenty-four hours about 1½ cm. rise of the vaseline cap is produced. The fluid clouds slightly and clears after eight days. This marks the stopping point of the reaction. There is no further production of gas nor is the cube of egg albumin reduced in volume or altered in any way. Hydrogen ion concentration after eight months, Ph. 6.8.

GELATIN

B. sporogenes. After twenty-four hours there is no reaction upon this media. Liquefaction starts after forty-eight hours, continuing gradually until the sixth day, when the gelatin is completely liquefied. Only a trace of gas is produced. The medium begins clearing on the eighth day, continuing to do so gradually. After sixty-five days the substance is clear.

A small amount of dirty gray residue is to be seen at the bottom of the tube. Hydrogen ion concentration at the end of eight months, Ph. 7.00.

B. botulinus. Complete liquefaction occurs after twenty-four hours with the production of 1 cm. rise of the vaseline cap in the tube. The fluid remains tenaciously cloudy until about thirty-five days have elapsed when it begins to clear. The fluid is entirely clear after about sixty-five days. The organisms settle to the bottom in a slimy, dirty gray residue. Hydrogen ion concentration after eight months, Ph. 7.00.

B. putrificus. No liquefaction is apparent until forty-eight hours have elapsed and at this time it is only partial, the stiff gelatin becoming reduced to a jelly-like consistency. After the third day the substance is completely liquefied. A trace of gas is produced. The very cloudy fluid clears gradually after twenty days, depositing the usual dirty gray residue upon the bottom of the tube. Hydrogen ion concentration at the end of eight months, Ph. 7.00.

B. oedematis maligni. On account of the powerful proteolytic action of this organism upon other media, it was somewhat of a surprise to note that its liquefying action upon gelatin was less rapid than that of other members of the proteolytic group.

There is no alteration after twenty-four hours, and after forty-eight hours there is only partial liquefaction in the depths of the culture. This gradually extends upward, being accompanied by profuse clouding. After four days the gelatin is liquefied except for a small zone at the top of the tube, extending downward about 1 cm. There is gradual liquefaction of this unaltered area until the eighth day when this process may be said to be complete. After about ten days the fluid begins to clear. Three weeks later there is no further evidence of clouding. At the end of forty-eight hours a trace of gas, amounting to a few bubbles, is produced. Hydrogen ion concentration after eight months, Ph. 7.00.

B. histolyticus. This organism completely liquefies gelatin after twenty-four hours, forming a dense white cloud of growth. At the end of forty-eight hours there is a marked clearing of the fluid, especially at the top. This process continues rather rapidly as compared with other members of the proteolytic group, and after about twelve days there is no further evidence of clouding. No gas is produced at any time. The precipitate accumulated at the bottom of the culture is nearly snow-white, while in the case of other members of the proteolytic group, as has already been pointed out, the residue takes on a dirty slate gray color. Hydrogen ion concentration after eight months, Ph. 7.4.

B. bifermentans. After twenty-four hours, partial liquefaction of the gelatin occurs, increasing to complete liquefaction after the second day. A small amount of gas accompanies this reaction. There is a marked tendency toward clearing after about the third week. At this time the top half of the medium is perfectly clear, while the lower portion is still cloudy. This clouding gradually disappears and after about thirty-five days the medium is perfectly clear. There is al arge amount of dirty gray residue at the bottom of the tube, composed mostly of spores and dead bacilli. Hydrogen ion concentration at the end of eight months, Ph. 7.00.

B. bellonensis. Although growth in this medium is profuse, there is no evidence of liquefaction until the eighth day, when partial liquefaction is to be noted. This condition remains unaltered for twenty-one days, after which time the gelatin may be said to be completely liquefied. Only a few bubbles of gas are produced. A characteristic of the reaction is the

slow action of the liquefying ferment. The fluid clears in thirty-five days, with the usual accumulation of dirty gray detritus at the bottom of the tube. Hydrogen ion concentration at the end of eight months, Ph. 7.00.

B. aerofoetidis. Growth in this medium is profuse after twenty-four hours, but there is no evidence of liquefaction until the end of the sixth day. This increases gradually until the eighth day when the process may be said to be complete. There occurs also at this time a clearing of the fluid. A few bubbles of gas are produced forty-eight hours after planting. Hydrogen ion concentration after eight months, Ph. 6.6.

B. oedematiens. Twenty-four hours growth in this medium is marked by the production of enough gas to cause about a 2 cm. rise of the vaseline cap in the tube. There is profuse clouding of the gelatin but liquefaction does not begin until the third day, becoming complete by the fourth day. Gas production after about eight days has slightly increased, amounting to over 3 cm. A small amount of white detritus is to be seen at the bottom of the tube, occurring simultaneously with the clearing of the medium which takes place after the eighteenth day.

Vibrion septique. The action of Vibrion septique in gelatin is complete after four days. The medium is thoroughly liquefied after twenty-four hours, accompanied by the production of enough gas to produce a 1 cm. rise of the vaseline cap. After forty-eight hours the amount of gas is doubled. Absolute clarification occurs on the fourth day. No further production of gas is noted. There is a sparse accumulation of whitish residue at the bottom of the tube. Hydrogen ion concentration, Ph. 6.4.

B. of Ghon and Sachs. The reaction in gelatin is identical with that described for Vibrion septique.

B. fallax. This is the only organism in the group studied which does not liquefy gelatin. Observations were made over a period of eight months and although there was rather luxuriant growth, no evidence of liquefaction was to be obtained.

B. welchii. Twenty-four hours growth in gelatin gives rise to the production of sufficient gas to cause the rise of the vaseline cap in the tube for about 2 cm. Complete liquefaction also occurs at this time. No further production of gas is noted and after six days the fluid becomes perfectly clear. Ph. 6.4.

B. tertius. In gelatin the action of B. tertius is in marked contrast to that produced by B. welchii. B. tertius produces a 2 cm. rise of the vaseline cap in the tube after forty-eight hours, and although growth is luxuriant, there is no evidence of liquefaction until after forty days, at room temperature. The process of liquefaction then continues slowly, being completed only after the forty-fifth day. Two days later the erstwhile cloudy broth is rendered clear. There is no further gas production. A small amount of white residue is deposited at the bottom of the tube. Hydrogen ion concentration after eight months, Ph. 6.6.

ALKALINE EGG FLUID OF ROBERTSON [9]

B. sporogenes. This organism clots the egg fluid during the first twenty-four hours, with the production of enough gas to cause a 1 cm. rise of the vaseline cap. The clot is subsequently rapidly digested. Even after twenty-four hours some proteolysis is to be noted at the top of the clotted egg fluid, and after forty-eight hours the material is about one-third digested, the proteolyzed portion being displaced by a turbid fluid. After the eighth day the clot is one-half digested and on the tenth day there are only a few small fragments to be seen floating at the top of the much clouded fluid. There is no further action. The fluid becomes somewhat clearer but even after eight months it remains slightly opaque. There is no further production of gas after the first twenty-four hours. The odor produced in this medium while characteristically foul is not as penetratingly so as that produced in other media. Hydrogen ion concentration after eight months, Ph. 7.00.

B. botulinus. B. botulinus acts with comparative slowness on the alkaline egg fluid, no effect being in evidence until the eighth day of incubation when feeble gas production becomes apparent. At this time also the fluid clots and there is a slight separation of the clot from the sides and top of the tube. The clot slowly digests, maintaining all the while its cylindrical appearance. Due to the fact that there is but feeble gas production it is not split as are the clots formed in the case of some of the other organisms. After thirty days incubation the clot is about one-half reduced in size, the residual portion still being in the shape of a cylinder. At this time also the fluid which has displaced the proteolyzed portion of the clot is water-clear. No further evidence of digestion is noted even after eight months observation. Hydrogen ion concentration after eight months, Ph. 6.8.

B. putrificus. As regards proteolysis, B. putrificus acts more rapidly on the alkaline egg fluid than on any other media. After twenty-four hours in the incubator sufficient gas is produced to account for a 1 cm. rise of the vaseline cap. The fluid has not only become thoroughly clotted but at the upper portion of the clot marked digestion is to be noted. At the end of 4 days the clot is reduced about one-half in volume and after 6 days have elapsed only about one-third of the substance remains. From this time on proteolysis proceeds gradually and after ten days in the incubator nothing remains of the clot, save a few brownish granules. The fluid which has displaced the digested substance is water-clear. There is no further production of gas. Hydrogen ion concentration at the end of eight months, Ph. 6.8.

B. tetani. This medium is but feebly attacked by the above-named organism. Aside from the egg fluids forming into a soft mushy clot after seventy-two hours incubation, there is no action until after about thirty days in the incubator. At this time evidence of very feeble proteolysis may be noted at the top of the mushy, fragile clot, leaving a narrow band of clear watery fluid between the clot and the vaseline. (This may also

be due to mere shrinking such as is noted in the case of organisms comprising the saccharolytic group.)   Proteolysis, if it is such, proceeds very slowly, varying in degree but little from week to week.   There is no gas formed at any time during the reaction.

After eight months there is a gradation in the tube from a narrow band of slightly opalescent fluid at the top to a lower zone of very loosely organized clotted material, there being in the central portion of the tube a condition about midway between a very heavy opalescent and a very loosely organized clot.   Only a faint odor of putrefaction is to be noted. Hydrogen ion concentration after eight months, Ph.˙6.9.

B. oedematis.   The action of this organism upon alkaline egg fluid strongly resembles that of B. sporogenes.   After twenty-four hours, sufficient gas is produced to account for the rising of the vaseline cap about 1 cm.   The fluid is thoroughly clotted and clear evidence of proteolysis is to be noted even at this early period.   Twenty-four hours later only about one-half of the clot remains and on the eighth day the residual portion amounts to about one-third of the original.   Water-clear fluid replaces the clot as digestion proceeds.   At the end of fourteen days the clot is entirely digested, leaving only a few small whitish fragments floating at the top. There is no further alteration.   Hydrogen ion concentration, Ph. 6.8.

B. histolyticus.   There is no action on alkaline egg fluid until after eight days of incubation.   A soft fragile clot is then to be noted but there is no gas produced nor is there any evidence of digestion.   On the tenth day a slight zone of proteolysis is in evidence at the top of the clotted fluid, with a narrow band of clear fluid overtopping it.   After fourteen days the clot is proteolyzed to the extent of one-half of its volume, crystal clear fluid replacing the digested portion.   The remaining clot consists of a network of fine filaments.   This marks the final reaction, as there is no further alteration.   Hydrogen ion concentration after eight months, Ph. 7.00.

B. bifermentans.   After twenty-four hours the vaseline cap is lifted about one-half cm. from the surface of the fluid.   There is a marked thickening òf the medium, causing it to appear about the consistency of heavy cream.   For the next nine days there is no alteration.   On the tenth day, however, the thickened fluid becomes frankly clotted with a trace of digestion apparent at the top.   Digestion gradually increases until the fourteenth day when the clot is about one-quarter diminished in size, surmounted by clear watery fluid.   There is no further alteration. Hydrogen ion concentration after eight months, Ph. 6.9.

B. bellonensis.   Aside from a thickening of the fluid after forty-eight hours incubation there was no further action upon this medium.   Hydrogen ion concentration after eight months, Ph. 6.9.

B. aerofoetidis.   The above mentioned organism gave rise to no reaction upon this medium.

Vibrion septique. Action upon this substance is rather characteristic in that after twenty-four hours the vaseline cap rises to the extent of 1 cm. The egg fluid daily becomes more opaque and at the end of fourteen days a soft mushy clot is formed. There is no separation at the top or sides, nor is there any evidence of digestion. Hydrogen ion concentration after eight months, Ph. 6.5.

B. of Ghon and Sachs. As for Vibrion septique.

B. fallax. As regards the action of this organism on alkaline egg fluid, my results are at variance with those obtained by Henry,[14] as in my hands B. fallax persistently refused to grow in the substance although repeated attempts were made to induce it to do so.

B. welchii. No action was observed in this medium, save the production of sufficient gas to account for the vaseline caps rising 1 cm. In my hands the cultures of B. welchii gave rise to no clotting or production of a fibrous network such as described by Henry.[14]

B. tertius. Regarding the action of B. tertius in alkaline egg fluid, my results agree with those obtained by Robertson[9] who found that B. tertius did not act upon the undiluted egg fluid. I am therefore at variance with Henry on this point for he described opacity and clotting after five days.

MODIFIED EGG WATER

The latency of action upon the unmodified alkaline egg fluid, and in some cases lack of any action at all, is no doubt due to the highly alkalinized condition of the medium. When the hydrogen ion concentration is so adjusted as to bring it nearer the optimal, the end reaction is reached in a much shorter time, and in the case of the organisms which did not act upon Robertson's medium at all, there is usually clotting and other evidence of activity upon the more nearly neutral fluid.

In preparing this medium, instead of adding 6 c.c. of the alkali directly to the substance as advised by Robertson, the eggs are added directly to the water, and the mixture is so titrated so as to bring its reaction to Ph. 7.2. Some of the albumin will, upon autoclaving, be found to have settled to the bottom of the tube, but there is sufficient left in solution to bring about a well defined action.

The reactions as regards B. sporogenes, B. botulinus, B. oedemaitis maligni, B. histolyticus, B. tetanus, and B. putrificus are essentially the same as has already been described under the heading of the more alkaline medium. The action of the other organisms differ somewhat and will therefore bear description.

B. bifermentans. There is no action upon this media until after forty-eight hours incubation. At the end of that time a soft clot is produced together with a rise of the vaseline cap, amounting to 1 cm. There is separation of the clot at the top of the tube, the clot being surmounted by a thick turbid fluid. Seventy-two hours of incubation gives rise to

digestion amounting to about one-quarter of the total volume of the clot, the digested portion being displaced by thick turbid fluid. The clot does not maintain its entirety, being flaky at the top, and but loosely organized throughout. Digestion and gas production proceed no further.

B. bellonensis. The fluid is solidly clotted after twenty-four hours. A slight trace of gas is produced but no evidence of digestion is to be noted at any time. There is no further reaction after twenty-four hours.

B. aerofoetidis. After twenty-four hours there is produced a trace of gas and a soft mushy, loosely organized clot. There is feeble evidence of digestion at the top of the clot.

B. oedematiens. A soft mushy clot is produced at the end of twenty-four hours. There is slight separation at the top and sides, the substance being replaced by clear fluid. The clot is at this time slightly split. After seventy-two hours the clot becomes more firm and is markedly shrunken. At this point gas is produced to the extent of a 1 cm. rise of the vaseline cap. There are no further reactions.

Vibrion septique. A soft mushy clot, after twenty-four hours incubation, with gas production to the extent of 1 cm. rise of the vaseline cap. After forty-eight hours, the clot becomes firm and tough and at the end of seventy-two hours incubation it is markedly torn and rent by gas. After seventy-two hours the clot has also markedly shrunken from the sides and top, being displaced by clear watery fluid. There is no digestion apparent.

B. of Ghon and Sachs. Essentially the same as Vibrion septique.

B. fallax. Aside from the production of sufficient gas to account for the vaseline cap's rising ½ cm., there is no reaction upon this media.

B. welchii. There is marked clotting of the media after twenty-four hours, together with production of gas causing the vaseline cap to rise 1½ cm. The clot is separated from the tube at the top and to a slight extent at the sides, being displaced by clear watery fluid. After forty-eight hours the clot becomes much shrunken, hard and caseous in consistency, the gas production is about doubled and watery fluid is copiously produced.

B. tertius. This organism does not alter the medium in any way.

INSPISSATED SERUM

B. sporogenes. After forty-eight hours incubation the coagulated serum will be found to have become markedly split and darkened to a dirty slate gray color. The vaseline cap is raised 2 cm. On the third day the substance is practically two-thirds digested and the fluid is decidedly clouded. Four days after incubation only a few small fragments of much blackened substance remain and on the sixth day the coagulated serum is entirely digested. The fluid upon this medium clears very rapidly, there being no trace of clouding after ten days. Gas production does not

increase after the second day. The usual characteristic foul odor accompanies this reaction. Hydrogen ion concentration after eight months, Ph. 8.oo.

B. botulinus. The reaction of this organism upon the clotted serum is somewhat slower than that of B. spnrogeoes.

After twenty-four hours, enough gas is produced to lift the vaseline cap 1 cm. and at this time also, the clot is somewhat shrunken away from the sides of the tube. There is no change until the third day when the vaseline cap is lifted 3 cm. The clot is much blackened and split and evidence of proteolysis is to be noted, especially at the top.

On the sixth day the substance is about one-half reduced in volume and is overlaid with cloudy fluid. After ten days the serum is at least three-quarters digested. Four days later the medium is entirely digested, with the exception of a few small granules which are jet black in color. The fluid is at this time perfectly clear. Hydrogen ion concentration after eight months, Ph. 7.2.

B. putrificus. The action of this organism on the clotted serum somewhat resembles that of B. tetani but as will be seen, it differs greatly from the action of members of the strongly proteolytic group already described.

There is no change until the eighth day when the media becomes somewhat darkened at the top, accompanied by sufficient gas production to raise the vaseline cap about 0.75 cm. After ten days incubation evidence of feeble proteolysis is to be noted at the top of the medium, and gas production about doubles. Digestion embraces about one-half of the clot at the end of two weeks and from this time on there is no further change. The turbid fluid clears some five days later. Hydrogen ion concentration after eight months, Ph. 7.4.

B. tetani. This organism does not give evidence of any activity until after six days of incubation when gas is produced, amounting to a few bubbles, and the substance turns slate gray in color. The darkening here produced is not nearly as intense as in the case of other organisms. There is no further change until the eighteenth day when the medium becomes somewhat split due to the additional amount of gas formed and there is evidence of feeble proteolysis to be noted, as the material is about twenty-five per cent reduced in volume. After twenty days in the incubator digestion only slightly increases. The remaining portion has become separated from the walls and top of the tube, being displaced by clear fluid. The odor here, as in the case of B. putrificus, is only very slightly putrefactive. Hydrogen ion concentration after eight months, Ph. 7. 4.

B. oedematis maligni. Probably the most rapid proteolytic action encountered was the complete digestion of this medium by the above-named organism in seventy-two hours.

There is no evidence of activity until the second day, when slight darkening of the substance is noted, accompanied by sufficient gas to raise the vaseline cap 1 cm.

After seventy-two hours the amount of gas is tripled and the coagulated serum is entirely digested, with the exception of a few small black granules, about the size of a pea, which are to be seen in the bottom of the tube. The turbid fluid clears on the tenth day.

Inspissated serum is a favorable medium with which to differentiate B. oedematis maligni from other members of the strongly proteolytic group. Hydrogen ion concentration after eight months, Ph. 7.4.

B. histolyticus. After forty-eight hours there is marked blackening of the medium together with separation from the walls of the tube. Twenty-four hours later the serum is about one-half digested, being overtopped with turbid fluid. After ten days the medium is about two-thirds reduced in volume but there is no production of gas. The remaining fragments of undigested serum assume a jet black color and after twenty-one days feeble gas production is noticed — about three or four bubbles. There is no further evidence of proteolysis, even after eight months.

Curiously enough the fluid of this culture remains tenaciously turbid, clearing only at the end of the third month, in marked contrast to other members of the strongly proteolytic group which usually clear upon this medium after ten to twenty days. After four months at room temperature a few characteristic balls of tyrosine are to be seen, mixed with the black residue. Hydrogen ion concentration after eight months, Ph. 7.9.

B. bifermentans. On the second day there is a marked darkening of the medium, together with the production of sufficient gas to raise the vaseline cap 2 cm. The coagulated serum is at this time somewhat shrunken, being displaced by clear watery fluid at the top, sides and bottom. On the third day the clotted serum is much split and markedly darker in color. It now has assumed a dirty slate gray hue. Four days later some evidence of proteolysis is to be noted. This proceeds slowly from day to day. After ten days incubation, the fluid, which has displaced the serum shrunken away from the walls of the tube, clears; at this time also the coagulated serum is about twenty-five per cent digested. After forty-five days the medium is probably about one-third digested, the remaining portion being much blackened and fragmented. After eight months the substance is reduced to about one-half of its volume, overtopped with a slightly acid fluid having a hydrogen ion concentration of Ph. 6.8.

B. bellonensis. This organism does not cause any change upon the inspissated serum until after six days of incubation, when the medium becomes shrunken away from the walls of the tube, darkened to slate gray color and split by gas which at this time raises the vaseline cap 1 cm. Aside from the fact that the dark color of the medium becomes more intense, there is no further action. After eight months the hydrogen ion concentration of the culture is Ph. 6.6.

B. aerofoetidis. After six days incubation the coagulated serum becomes shrunken away from the walls of the tube, there is sufficient gas produced to lift the vaseline cap 1 cm., and the medium becomes slightly

darkened. The color here produced, however, is not nearly as intense as that produced by organisms whose action upon this medium has already been recorded. After ten days the serum becomes much softened and gas production about doubles. Clear fluid is to be noted where the medium has separated from the walls of the tube.

After three months the evidence of very feeble digestion is to be noted at the top of the medium, and the darkening of the substance becomes somewhat intensified. Aside from this there is no further alteration even after eight months, when the hydrogen ion concentration is Ph. 7.00.

B. oedematiens. After forty-eight hours in the incubator the clotted serum becomes somewhat separated from the walls of the tube. There is gas produced in sufficient volume to lift the vaseline cap 2 cm., but there is not even the faintest suggestion of digestion. This marks the end reaction. No further change was noted even though the cultures were incubated for forty-five days and kept under observation at room temperature for eight months. Hydrogen ion concentration after eight months, Ph. 6.8.

The cultures of B. tertius, B. fallax, Vibrion septique and B. of Ghon and Sachs act upon this medium in the same way as does B. oedematiens, and no further description of them is given. B. welchii, however, forms a large amount of gas, splits the medium into several fragments and forms more dark pigment than does any member of the non-proteolytic group. There is not, however, any evidence of digestion.

COOKED CODFISH MEDIUM

The reactions upon this medium are essentially the same as those upon cooked meat medium, therefore, a brief summary only is given.

B. sporogenes. Gas is produced amounting to a 4 cm. rise of the vaseline cap. After a lapse of a week the fish muscle is about three-quarters digested, and there is profuse production of tyrosine crystals. The tissue becomes much blackened during the process of digestion. There is the usual foul odor and tenacious turbidity of the broth. After eight months, a few black granules about the size of a pea remain. The broth at this time is clear. Hydrogen ion concentration, Ph. 7.0.

B. botulinus. Gas is produced sufficient to raise the vaseline cap 2 cm. Digestion of practically all of the fish tissue occurs during the first week, accompanied by blackening of the tissue and the production of a foul odor. The broth clears after fourteen days. At the end of eight months, a very small amount of black residue is to be seen at the bottom of the tube. Hydrogen ion concentration, Ph. 7.0.

B. putrificus. The vaseline cap is raised four cm. after the first twenty-four hours of incubation and after seventy-two hours the fish tissue becomes much darkened. When two weeks have elapsed, the tissue may be

said to have become reduced in volume about one-third. The odor produced in this reaction is not as penetratingly foul as that of B. sporogenes. After eight months the fish muscle is reduced about two-thirds leaving a dirty dark gray granular residue. No tyrosine crystals are produced upon this medium. Hydrogen ion concentration, Ph. 7.1.

B. tetani. After forty-eight hours incubation, gas is produced sufficient to raise the vaseline sale about 4 cm. The fish tissue becomes blackened in spots and there is a faint odor of putrefaction. Digestion upon this medium is not marked. After eight months only slight reduction is to be noted.

B. oedematis. The reaction of this organism upon the cooked codfish medium is identical with that of B. sporogenes, with the exception that no tyrosine crystals are produced.

B. histolyticus. The codfish becomes much darkened after twenty-four hours incubation. There is no gas produced, however, throughout the entire reaction. After a week the tissue is digested about one-third, and the fish muscle becomes black in color. Proteolysis increases gradually, until after eight months the tissue is reduced about two-thirds in volume. The characteristic balls of tyrosine are produced upon this substance in such profusion as to nearly obscure the fish tissue. Digestion is here carried to the ammonia stage. Hydrogen ion concentration, Ph. 8.4.

B. bifermentans. After forty-eight hours incubation, the codfish assumes a dark, slate gray color, and gas is produced sufficient to raise the vaseline seal about 2 cm. The fluid becomes very turbid. Proteolysis upon this medium is very feeble. After eight months there is probably twenty-five per cent reduction of the tissue. Hydrogen ion concentration, Ph. 7.1.

B. bellonensis. Aside from the production of gas sufficient to raise the vaseline cap about 2 cm., and a slight darkening of the fish muscle, there is no marked reaction upon this medium. Hydrogen ion concentration after eight months, Ph. 7.2.

B. aerofoetidis. The fish tissue becomes slightly darkened after forty-eight hours of incubation, and at this time also a few bubbles of gas are produced. There is no further reaction. Hydrogen ion concentration after eight months, Ph. 6.9.

B. oedematiens. Aside from the production of gas sufficient to raise the vaseline seal 1 cm., there is no further action upon this medium.

Vibrion septique, B. of Ghon and Sachs, B. fallax, B. welchii, and B. tertius act identically upon the cooked codfish medium in that they all produce a small amount of gas, but do not alter the tissue in any way.

PRODUCTION OF HEMOLYSINS

The method of Lyall [15] was used to determine which of these anaerobic spore-bearers produced hemolysins. This technic has the advantage of being accurate as well as readily applicable to this type of organism, and gives one a good quantitative estimate of the hemolytic agents produced.

The bacteria were grown in broth made according to Lyall's formula; in addition, however, was added two per cent of the

TABLE II. PRODUCTION OF HEMOLYSINS

	0.1 cc	0.2 cc.	0.3 cc.	0.4 cc.
B. sporogenes.......	o	o	Methemoglobin	Methemoglobin
B. botulinus	o	o	Methemoglobin	Methemoglobin
B. oedematis	o	o	Methemoglobin	Methemoglobin
B. histolyticus	o	++	+++	++++
B. putrificus	o	o	Methemoglobin	Methemoglobin
B. tetani	++++	++++	++++	++++
B. bifermentans.....	o	o	o	o
B. bellonensis.......	o	o	++	+++
B. aerofoetidis	o	o	o	o
B. oedematiens	o	o	o	o
Vibrion sept.	+	++	+++	++++
B. Ghon-Sachs......	o	+	++	+++
B. fallax	o	o	Methemoglobin	Methemoglobin
B. welchii	++	+++	++++	++++
B. tertius	+	++	+++	++++
Control-Ph. 7.2	o	o	o	o
Control-Ph. 8.0	o	o	o	o
Control-Ph. 5.0	o	o	o	o

casein digest fluid, as some of the types refused to multiply upon the plain broth. The tubes were rendered anaerobic by the usual vaseline-seal boiling method. Luxuriant growth appeared at the end of twenty hours. At this time, to each of four serological tubes, was added, 0.1, 0.2, 0.3, and 0.4 c.c. of the broth culture of each organism, and 1 c.c. of a five per cent suspension of washed, fresh sheep erythrocytes. Enough physiological salt solution was then placed in each tube to bring the total volume up to 2 c.c. Thus in the case of each

organism, four separate tests were made. The tubes were incubated in a water bath for one hour at 37° C. and at the end of that time readings were made.

The results of the hemolytic action of these bacteria are reported in Table II.

MORPHOLOGY

The morphology of the anaerobic spore-bearing bacteria has been described by many observers in reference to a number of different media and under varying degrees of anaerobiosis. The bacteria comprising this group seem especially sensitive to their environment, and changes of type of culture media employed, relative anaerobiosis obtained and age of culture are all factors sufficient to influence the size and shape of these organisms. Therefore as regards morphology, much confusion has resulted.

Adamson [40] advocated grouping the anaerobic spore-bearers according to the shape and position of the spore. In my opinion this method is unreliable as the shape, and especially the position of the spore, varies to a considerable degree with the factors mentioned above.

To prevent differences in morphology being due to environmental conditions, all of the species described in this comparative morphological study were grown upon the same type of medium, and allowed to develop under a constant degree of anaerobiosis. Anaerobiosis was induced by the usual vaseline cap boiling method.

Some of the organisms of the non-proteolytic group refused to sporulate, and experiments were devised to obtain a medium which would induce sporulation in the minimum length of time. The formula for the stiff sugar-free agar found effective for this purpose is as follows:

To a liter of beef-infusion broth add 50 c.c. of casein digest fluid. This is inoculated with a strain of B. coli and incubated for twenty-four hours. Heat in the Arnold for thirty minutes at 100° C. after having added 25 grams of agar. Adjust to Ph. 7.4, filter, tube and sterilize. The cultures were incubated for forty-eight hours at 37° C. and allowed to stand in the ice box for an additional twenty-four hours. They were then

stained by Gram's method and examined. Every one of the anaerobes, including B. welchii and B. tetani, had formed spores at the end of the seventy-two hour period.

Morphological Descriptions are as Follows

B. sporogenes. A medium sized gram-positive bacillus varying in length from 1.8 to 4.5 micra, the average being about 2.8. The width is constant, from 0.8 to 0.6 micra. The organisms for the most part occur singly and in pairs, a few short chains of from 7 to 10 are sometimes found. Ends are rounded, spore-bearing forms are very numerous. Organisms are always greatly swollen at the site of the spore. Spores vary in position from terminal, through subterminal to central. Even the organisms containing central spores are swollen at the position of the spore. Sub-terminal sporulating forms are by far the most numerous.

The spore is usually oval, but a few rounded forms were noted. Size rather constant, measuring about 1.8 × 1.0 micra. The spore is very large with exceptionally thick walls.

B. botulinus. A large, thick, gram-positive bacillus with rounded end. The spores are for the most part situated subterminally while a few are central. Upon this medium the organisms bearing spores retain the gentian violet with great tenacity while the vegetative forms seem to have lost this power to a considerable degree. Spore production is accompanied by a swelling of the organism, not only at the point of sporulation but of the entire bacillus. The vegetative forms are much more slender than those organisms showing spores. Vegetative forms from 2.0 to 5.0 micra long average about 2.5, width, from 0.3 to 0.5 micron. Spore-bearing forms measure in length from 2 to 9 micra, the average being about 4 micra. The width is rather constant, measuring from 0.5 to 0.7 micron. While spore-bearing types are numerous, no free spores were to be noted.

B. putrificus. This bacillus stains irregularly, taking to some degree the counter stain, thus giving it a peculiar mulberry-like color. The organism is small, of medium thickness, with rounded ends. Some forms show a pointed terminal structure. They occur singly, in pairs and in small groups. No chain forms were noted. Some of the forms have a peculiar banded appearance due, no doubt, to the irregularity with which the organism takes the stain. The spores are oval in shape and situated terminally in most cases; however some of the forms show the spores to be slightly subterminal in position. Those organisms showing a well defined spore nvariably had a small bit of bacterial protoplasm situated at the sporulating end, giving a peculiar capped, or spiked appearance. The size of the organism varies from 1.00 to 4.00 micra, the average being about 2.5. In width it measures from 0.3 to 0.4 micron.

B. tetani. This organism does not sporulate as profusely as do the other bacteria, even upon this sugar-free medium. Quite a few spore-bearing forms are to be noted, however, after seventy-two hours. B.

tetani is large and gram-positive, measuring in length from 2.0 to 6.0 micra the average length being about 3.0 micra. The width is about 1.0 micron. The spore which is usually situated terminally is large, and for the most part rounded. Some forms were noted which possessed slightly subterminally spores, rather oval in shape. Many of the forms stained with that irregularity which gives a banded or beaded appearance. The spores measure 1.0 × 0.8 micron. The ends are for the most part rounded or pointed. An occasional clubbed form is met with due to the usual swelling at the point of sporulation.

B. oedematis maligni. A large gram-positive bacillus with rounded ends, occurring singly, in pairs and in groups. No chain forms were noted. Spore is oval, large and terminal in situation. Occasional forms, however, with subterminal spores were noted. Some forms were encountered which stained irregularly, giving that peculiar banded or beaded appearance. Length of bacillus, from 1.5 to 3.5 micra, the average being about 2.5. Width, from 0.3 to 0.4 micron. Size of spore, 1.5 × 0.5 micra. Morphologically, B. oedematis strongly resembles B. putrificus.

B. histolyticus. A small thin bacillus with rounded ends. This organism does not retain the gentian violet very tenaciously, seeming to have somewhat of a gram-negative tendency. The forms occur singly, in pairs and in groups; no chain forms were noted. The spores are large, oval and terminal in situation, being often much longer, and invariably wider than the bacillus. Length of bacillus, from about 0.8 to 2.0 micra, average about 1.7. Width, about 0.3 micron. The spore measures about 1.2 × 0.5 micra. It is interesting to note the wide variation of B. histolyticus from the above description when planted in a liquid medium. The organism is much longer and thicker, averaging in length about 2.5 micra and in width about 0.7 micron. The spores are for the most part situated subterminally, in some cases being central in position. The spores are also much smaller, measuring about 0.3 × 0.6 micron. On the liquid medium B. histolyticus varies from a thin bacillus with a large subterminal spore to a stout oval organism with the spore central in position, and exhibiting a marked swelling at the point of sporulation.

The other representatives of this group of sporulating anaerobes show similar morphological variations when grown upon various media, indicating how futile it is to attempt classification according to the position of the spore, and how necessary it is to use the same kind of medium for all of the species when one desires to describe their morphology.

B. bifermentans. A large thick gram-positive bacillus strongly resembling B. bellonensis but shorter and thicker. The organisms occur singly, in pairs and in short chains of from three to seven. The bacilli are for the most part straight with square or very slightly rounded ends. An occasional curved form is, however, to be seen. The spores are large and usually situated centrally, some few being subterminal. B. bifermentans varies in length from 2.0 to 4.5 micra, with the large majority of organisms measuring about 2.5 micra. The width averages about 1.0 micron.

The spores are large, having rather sharply rounded ends, giving one the impression of being oblong rather than oval. They measure 1.5×0.5 micra. No swelling or enlargement at the point of sporulation is to be noted.

B. bellonensis. This bacillus is large and thick with rounded ends, and frankly gram-positive. They occur singly, in pairs, and in chains of from three to ten. The length varies from 2.5 to 7.0 micra, the average being about 4.0 micra . The width is about 0.7 micron. Most of the organisms are straight rods but a few curved forms taking the shape of the letter C or S were to be seen. The spores are usually situated centrally, with a few subterminal. The spores measure about 1.2×0.5 micra. Quite a few of the organisms were occupied almost completely by the large central spore, leaving only a small discernible outline of the bacillus.

B. aerofoetidis. A short plump gram-positive bacillus with rounded ends, occurring singly and in pairs. No chain forms were noted. The organism measures in length from 0.8 to 2.5 micra, the average being about 1.8, the width varying from about 0.5 to 0.7 micron. The spores are usually situated subterminally, and are small, measuring about 0.8×0.4 micron. Spore-bearing forms of B. aerofoetidis were not numerous.

B. oedematiens. A medium sized gram-positive bacillus with rounded ends. The organism, although retaining the gentian violet, does so in an irregular manner, giving rise to somewhat of a mottled appearance. These forms occur singly and in pairs and groups. No chain forms were seen. The size is constant, not varying a great deal from 3.0×1.0 micra. The spore is oval, measuring about 1.0×0.5 micron and is invariably situated subterminally.

Vibrion septique. A slender gram-positive bacillus with rounded or pointed ends. It occurs singly, in pairs, and in short chains of from three to twelve. A few curved, swollen forms resembling the letter C were noted. Some of the individuals stain irregularly giving the peculiar beaded appearance. Vibrion septique varies in size from 1.5 to 7 micra, the average size being about 2.5 micra. The width is from about 0.3 to 0.4 micron. The spores are usually terminal. There are, however, occasional individuals having subterminal spores. The spores are relatively large, and oval, measuring about 1.5×1 micra. The spore-bearing forms of Vibrion septique remind one strongly of a short handled tennis racket.

Bacillus of Ghon and Sachs. Essentially the same as Vibrion septique.

B. fallax. A small, slender bacillus with positive gram staining properties. Some of the individuals retain the gentian violet but lightly however. The spores are usually situated terminally, with an occasional subterminal form. B. fallax usually occurs singly, in pairs, and in groups.

No chain forms were observed. In the non-sporulating forms, the organism is very thin, the ends are pointed, giving rather a characteristic appearance of tapering toward each end. The length varies from 1.5 to 3.0 micra, a large number of individuals measuring 2.0 micra. Width, from 0.2 to 0.3 micron. The spores are usually round and measure 1 micron in diameter.

B. welchii. As is well known, this organism measures from 4 to 6 micra in length and about 0.7 micron in width. It is gram-positive, and occurs singly, in pairs and in short chains of from three to twelve. The ends are for the most part square, with some individuals having rounded terminal structure. The morphology on the whole is very regular. The spore is usually situated centrally, with some few having subterminal spores. At times the spore is very large, taking up most of the bacillary body. It measures about 1.5 × 0.5 micra.

B. tertius. On this medium B. tertius is long and slender, with rather square ends. The organism is gram-positive but does not stain very deeply with the gentian violet. It occurs singly, for the most part; no chains and but few pairs were noted. The spore-bearing forms are usually long and slender, while the vegetative forms are relatively short and thick. The length of the spore-bearing types is from 3 to 6.0 micra, the majority measuring about 4 micra. The width is about 0.4 micron. The non-spore bearing forms vary in length from 2.0, to 3.5 micra, and are about 0.5 micron wide. The spore is usually terminal in position, and round, measuring about 1 micron in diameter.

CONCLUSIONS

From the study reported we feel that the following conclusions are justified:

1. The Barber single cell technic is the most feasible method of obtaining anaerobic spore-bearing bacteria in *pure culture.*

2. The vaseline-seal boiling method is the most reliable way of obtaining anaerobiosis, especially in liquid media. The technics now commonly used, involving the use of pyrogallic acid and sodium hydroxide, vacuum exhaustion, hydrogen displacement, and platinized carbon, were found to be unreliable.

3. If the proper degree of anaerobiosis is maintained the bacteria comprising this group will grow well on relatively simple media, provided the hydrogen ions are adjusted to the optimal degree of concentration, which was found to be Ph. 7.2.

4. A certain number of the organisms studied produced no proteolysis on any of the media and are therefore classified as *non-proteolytic* spore-bearing anaerobes. They are the following:

> B. welchii
> B. tertius
> B. fallax
> Vibrion septique
> B. oedematiens

5. Of those organisms studied which produced proteolysis, two groups may be made, the strongly proteolytic and the feebly proteolytic. They are as follows:

Strongly proteolytic	Feebly proteolytic
B. sporogenes	B. putrificus
B. botulinus	B. tetani
B. oedematis maligni	B. bifermentans
B. histolyticus	B. bellonensis
	B. aerofoetidis

6. B. putrificus and B. tetanus, although usually classified as strongly proteolytic, must on the basis of this study be placed in the feebly proteolytic group.

7. The reaction of these bacteria on the so-called proteolytic types of media may in some instances be of specific differential value. For the most part, however, these media serve to classify them into the three groups mentioned above. The absolute recognition of a species within a group can only be made by studying the carbohydrate fermenting properties of the bacteria.

8. Proteolytic activities of these bacteria are not lessened by the presence of fermentable carbohydrates.

9. The morphology of the anaerobic spore bearers studied, varies greatly with changes in environment and conditions of cultivation. When a constant medium and technic are employed, the morphology of any given organism remains only fairly constant. In the case of two or three species examined, however, the morphology was found to be very variable even when all conditions were kept the same. Morphology, therefore, offers but little aid in differentiation.

10. The shape and appearance of the colony does not form a reliable basis upon which to classify these bacteria.

11. The hemolytic properties of these organisms may in some cases be of differential value.

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Chambers' Micromanipulator for the Isolation of a Single Bacterium

MORTON C. KAHN

CHAMBERS' MICROMANIPULATOR FOR THE ISOLATION OF A SINGLE BACTERIUM

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As I have been working for several years on the isolation of bacteria with Barber's apparatus and have recently been using Chambers' instrument for the same purpose, Dr. Chambers suggested that I make some comments on the relative merits of the two instruments together with a short review of Barber's isolation method.

The mechanical principles involved in the construction of this instrument are entirely different from those of Barber's pipet holder, while the basic methods of manipulation are essentially the same. There are several noteworthy features in the Chambers' instrument which modify and improve the original Barber technic. These features may be best described under two headings: (1) advantages in construction, and (2) advantages from the use of various accessories to aid in manipulation.

ADVANTAGES IN CONSTRUCTION

The mechanical principles on which the instrument is based are fully described in the preceding article by Chambers.[1] The absence of parts which may loosen by wear and tear renders possible great precision in the manipulations. While excellent work may be done with the Barber pipet holder, the parts wear somewhat after several months' use, giving rise to a certain amount of false motion. For instance, when one desires to move the pipet laterally one may encounter an unexpected vertical motion. The Chambers apparatus used by me had been in use for two years, and in spite of this I was unable to detect any false motion.

A second advantage is that the instrument clamps directly on the stage of the microscope giving much greater rigidity than is possible with the metal flange which has to be attached to the stage of the microscope when Barber's instrument is used. Third, the smaller size of the instrument brings all manipulations closer to the microscope and

eliminates accidental jostling of the pipet holder which may shift the
needle out of focus. This compactness also makes it feasible to use a
short pipet which is easier to focus and is in less danger of contamina-
tion as less of the pipet is exposed.

ADVANTAGES OF THE ACCESSORIES IN CHAMBERS' APPARATUS

Barber's instrument possesses no accessories so that all the adjust-
ments, both preliminary and operative, have to be made by means of the
same finely threaded screws with a consequent waste of considerable
time.

In Chambers' instrument the several accessories are as follows:

1. There is a brass collar (fig. 2′) through which the shank of
the pipet is inserted before clamping it in the pipet carrier of the
instrument. Besides insuring rigidity to the pipet and thus greater
accuracy for manipulation, the collar facilitates bringing the pipet into
the field of the microscope. It also steadies the pipet as it is being
withdrawn from the moist chamber, thus minimizing contamination or
injury to the delicate tip.

2. For the vertical manipulation of the pipet there are three different
adjusting devices: first, the telescoping pillar for roughly adjusting
the pipet to the height of the moist chamber, after which it may be
tightly clamped; second, another coarse adjustment operated by a spring
screw with which one may bring the pipet into focus, and, third, the
fine adjustment of the knurl headed screw (fig. 1), which is used in
the actual operation of isolation. The first two devices are for the
coarse adjustment and enable one to use moist chambers of practically
any height. They aid greatly in the technic of the vertical adjustment,
which is the most important one from the bacteriologic point of view.

3. The use of levers on the screws controlling the lateral move-
ments insures great delicacy to the touch and is a decided aid in bring-
ing the pipet directly under the droplet containing the organism,
especially when working with the higher power or oil immersion lens
where the slightest movement is greatly magnified.

4. The flexible shaft attached to the vertical control screw brings
the movement of that screw behind the microscope away from all work-
ing parts and close to the fine adjustment of the microscope.

For bacteriologic work it is more convenient to have the moist
chamber as Barber originally devised it, viz., with its open end so
placed that the pipet may project into it from the left side. This

facilitates the frequent interchange of pipets so necessary in isolating bacteria. For this purpose it is necessary to have the pipet holder attached to the left side of the microscope. This can be done with the form which Chambers designates as right-handed by fastening it on the left side of the microscope stage near the outer corner (fig. 1).

In the preceding article it has been recommended to have the height of the moist chamber equal to the working focal distance of the sub-
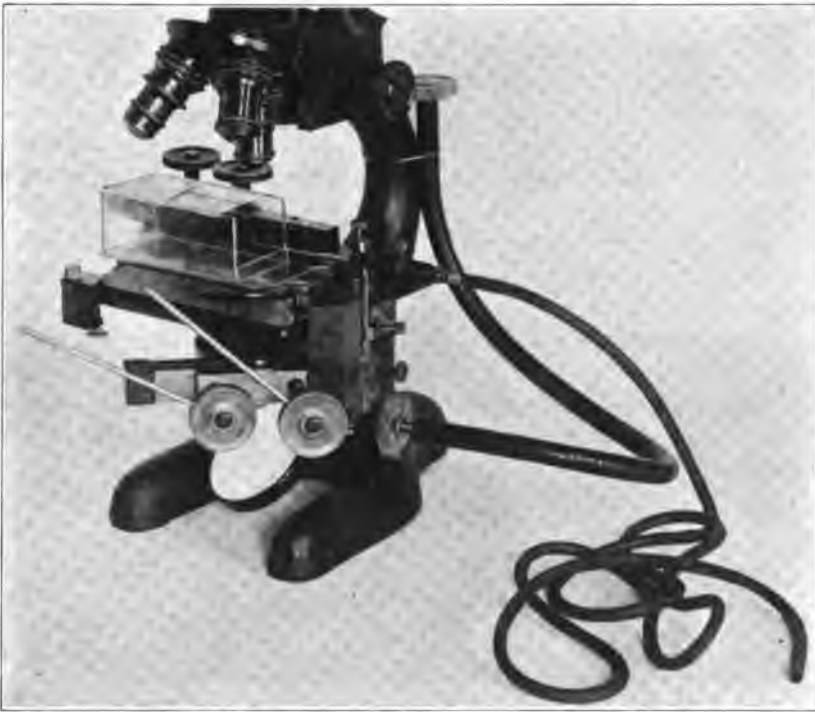
Micromanipulator mounted on the left side of the microscope for isolating bacteria. Note Barber's moist chamber with the coverslip marked with cross lines to aid in locating areas. The chamber shown here is higher than necessary.

stage condensor. This limits the height to one which may make the chamber too shallow to be convenient for frequent interchanges of the pipet. I, therefore, use a chamber ¾ of an inch high, 1 inch wide and 2½ inches long. It will be noted that this moist chamber is not only deeper but also wider than the one Chambers uses for cytological work.

THE ISOLATION METHOD

For the isolation of a single bacterium one must have the under sur-
face of the coverslip so treated as to hold minute droplets without fear
of their spreading or possibly running together. The droplets placed on
the coverslip must be slightly hemispheroidal in order that their outlines
may be distinct and all parts of it clearly visible. Also, in order to
maintain these droplets throughout the operation, the moisture condi-
tions within the chamber must be sufficient to prevent their evaporation
and at the same time must not be too great for fear of flooding them.

It is necessary, therefore, to have the surface of the coverslip
specially prepared. Barber, after smearing the cleaned coverslips with
petrolatum, washes them with soap and water to get rid of the excess
of petrolatum. The coverslips are then carefully cleaned with a dry
cloth, heated enough to soften the petrolatum and rubbed again while
still warm. The aim is to remove as much petrolatum as possible
without the use of excessive heat or any fat dissolving reagent other
than soap. If an excess of petrolatum is left on the cover, small
particles will appear in the droplets and may be mistaken for bacteria.
If all petrolatum is removed, the droplets run together and make
successful isolation impossible. Instead of soap and water one may use
95% alcohol with equally good results. One must realize that success
or failure in isolation work depends on a proper treatment of the
coverglass.

The method of procedure for the isolation of a bacterium may be
summarized as follows: [2]

1. Prepare a young liquid culture from a subculture not more than 18
hours old.

2. Insert the tip of a needle into a tube of the liquid culture and convert
the needle into a pipet by gently rubbing it against the wall of the tube. Then
with a rubber tube on its shank suck up a small amount of the culture.

3. Insert the pipet in the brass collar, then clamp it in the pipet holder of
the instrument and bring the tip into focus in the center of the microscopic field
(see figure). Raise the pipet until its tip touches the undersurface of the cover-
slip and expel an appreciable droplet. This may have to be diluted with sterile
fluid if the culture is too dense.

4. After securing a moderately dilute preparation fill the same or a new
pipet to a little below its bend. Lower the pipet, and with the aid of the
mechanical stage, bring another portion of the coverslip into view. By alter-
nately raising and lowering the pipet a series of minute droplets will be pro-
duced on the coverslip wherever the pipet touches it. The fluid runs out by
capillary attraction and needs no blowing. Some of these droplets will be
found to contain a single micro-organism.

[2] The method of making the pipets is described in the preceding article by Chambers.

5. Replace this pipet with a new sterile one containing a small amount of sterile liquid medium which must not run below the elbow. This new pipet is now brought directly under a droplet containing a single micro-organism. The pipet is then slowly raised and as soon as it touches the surface the droplet with the contained organism will flow into it. This occurs by capillary attraction and no suction is required.

6. This pipet, which is known to contain only one micro-organism, is carefully removed from the apparatus and its tip inserted into a tube containing a suitable sterile medium. The entire contents of the pipet are now to be expelled by blowing. As an added precaution it is well to break off the tip of the pipet in the culture medium. The blowing may be done by mouth or by a rubber bulb operated either by the hand or the foot.

VI. A SEROLOGICAL STUDY OF THE BACILLUS OF PFEIFFER

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I. THE ETIOLOGICAL RELATION OF THE BACILLUS OF PFEIFFER TO INFLUENZA

The bacteriological studies of the recent pandemic of influenza, also referred to as the "Spanish disease" and "grippe," have resulted in a divided opinion regarding the rôle of Pfeiffer's bacillus in the disease. The investigators have concluded either that this microörganism is the primary cause of the disease or that it is a secondary invader exerting more or less influence on the clinical course of the primary infection.

In Europe, opinion in this regard has been influenced by the degree of success that attended the efforts to cultivate *B. influenzae* from the sputum and organs of the affected individuals. Some failed entirely to demonstrate the organism and some found it in only a few cases. These workers (10, 12, 23) opposed Pfeiffer's view of the significance of his bacillus in influenza and some of them were inclined to the view—which was based on cultural studies and inhalation (spray) experiments in human beings with filtered sputum (1, 4, 26, 35)—that influenza is caused by a filterable virus. Another group of workers (2, 22, 41, 42) found a gram-positive "diplo-streptococcus" or a diplococcus and out of this finding comes a suggested etiological significance of these microörganisms. Other European investigators (8, 9, 16, 21, 24, 25, 26, 36) who isolated *B. influenzae* in a considerable

proportion of their cases (50 to 75 per cent) offered these findings
in support of Pfeiffer's view. A few writers question the use of
the negative findings as an argument against the etiological sig-
nificance of *B. influenzae*, on account of the known difficulty of
cultivating that microörganism.

The majority of American investigators have been inclined to
favor the view of Pfeiffer, either because of the production of a
pathological condition in monkeys resembling influenza by the
administration of cultures of *B. influenzae* (5); or because of
similar results with other lower animals together with the dem-
onstration of a toxin-like poison in broth cultures of *B. influenzae*
(7, 18, 29); or, finally, because of an apparent prophylactic action
resulting from the injection into human beings of "vaccine" pre-
pared from cultures of *B. influenzae* (6). Some (30), it is true,
are impressed by the frequent finding of *B. influenzae* in influenza.

On the other hand, the idea that the primary cause of influenza
is a filterable virus is not without followers in this country.

On the basis of extensive serological studies carried out under
his direction, Park (27, 28) has introduced a new argument into
the discussion of the question in hand. The argument lies in the
self-evident proposition that the most characteristic feature of
influenza; namely, its pandemic occurrence, which distinguishes
it from all other infectious diseases, requires the assumption of
a single etiological factor in its causation. According to this
proposition it is not sufficient, in order to prove the causal rela-
tionship of Pfeiffer's bacillus to influenza, that an obligative hemo-
phylic, pathogenic bacillus be found in all or nearly all cases of
influenza. It is necessary, in addition to this, to show, with the
aid of reliable biological reactions, such as that of agglutination,
that the cultures obtained from influenza patients that are known
not to have been in contact with one another are identical.

It was with this principle in mind that Eugenia Valentine and
Georgia M. Cooper (40), under Park's direction, made their
study of different cultures of *B. influenzae* obtained from cases of
influenza during the recent epidemic. These investigators in-
jected rabbits with cultures of *B. influenzae* and tested each indi-
vidual antiserum produced against a single culture with the same

(homologous) culture and with other cultures of *B. influenzae*. The tests were made by direct agglutination and by agglutination after absorption with the homologous cultures as well as with the other cultures that showed cross-agglutination.

The results obtained with the use of this method of study were surprising and highly illuminating. Not only were no identities of the cultures found, excepting where previous contact betwen the individuals concerned could be demonstrated or surmised, but in one family, the six members of which were stricken at very nearly the same time, the six cultures obtained were all different. Thus, even under circumstances most favorable for the finding of a hypothetical pandemic strain or culture of the bacillus of Pfeiffer, the study failed to reveal the existence of any such strain.

In the light of Park's argument these observations seem to admit of but one conclusion; namely, that Pfeiffer's bacillus cannot be the specific cause of influenza.

The importance of this conclusion makes it obviously advisable that the observations on which it is based be confirmed and it was with this purpose in view that the present study was undertaken.

The cultures studied were of six groups as to source.

Cultures Holmes, Eldridge, Gordon and Amert were of the marine group of Valentine and Cooper, who designated the cultures as M7, M3, M4 and M1, respectively. Cultures Leuchner and Meyer were the cultures H11 and H13 of Valentine and Cooper. Cultures Lee, Williams, Masates and Godfrey were autopsy strains designated with these names in the paper of Valentine and Cooper. Cultures Angelo and Michael were derived from the family group of Valentine and Cooper. Cultures 126, 159, 160 and Witt were isolated from cases of influenza during the winter 1919–1920 by Dr. Anna Williams. Cultures 62 and G. L. T. were kindly supplied by Dr. E. O. Jordan of Chicago, these having been isolated from cases of influenza in Chicago during the last winter.

Thus, the cultures subjected to the present examination were derived from widely different sources, both as to the time and as to the locality of their isolation.

CULTURE MEDIUM

Throughout the present study we have used the so-called "chocolate" medium; that is heated blood-agar. The proper method of preparing this medium and the history of the method seem generally to have had inadequate consideration in the literature.

The advantage of heating the mixture of blood and agar was first noted by Dr. Olga Povitzky in an inconspicuous remark made in the course of one of the Collected Studies from the Bureau of Laboratories, Department of Health, City of New York, 1912–13, vii, 93. At the foot of page 94 is found the following statement: "Recently we found that a more vigorous growth is obtained on coagulated blood medium. This medium is obtained by adding blood to agar (1:10 to 1:500) at 90°C."

In a paper entitled "Zur Züchtung des Influenzabazillus," Hundeshagen referred to an "American method" of which he had learned in 1915, but which he could not find in the collected studies referred to, although he had searched through the issues of the "past four years." Hundeshagen employed the method with very good results. He heated the blood-agar mixture at 96°C.

A. Fleming boils the blood-agar mixture for one minute and obtains an "enormous growth" in this slanted mixture.

In our experience the volume of growth of *B. influenzae* on the blood-agar medium is markedly influenced by the temperature to which the mixture has been heated. While no formal experiments upon this point have been carried out, we can say that the temperature which will barely cause the coagulation of the blood, with the production of the chocolate brown color, is not high enough to provide the optimum growth conditions in the medium; these conditions can be secured by subjecting the mixture to a temperature between 96° and 100°C. for a period of ten minutes—possibly less. The mixture can be made in individual tubes, these having been taken out of boiling water and returned to it for the required time; or the mixture can be made

in bulk in a flask immediately after the agar has been removed from the autoclave.

We have employed neutral, nutrient agar containing 5 per cent glycerin, adding 10 per cent of sterile horse's blood.

PRODUCTION OF ANTISERA

Twenty-four-hour cultures were injected intravenously into medium sized rabbits over a period of three weeks, as follows:

On the first day a suspension of one agar slant growth in 2 cc. of sterile saline solution was heated for one hour at 60°C. and of this 1 cc. was injected.

On the 11th, 12th, 13th, 17th, 18th, 19th and 20th days 1 cc. of a similar suspension of the unheated bacteria was injected. On the 25th day the animals were bled and the sera were tested with the method of direct agglutination. If the titer of the serum was found to be lower than 1:640, as it was in two instances, the injections were continued daily for a period of ten days, after which in both cases the titer was found to be satisfactory; that is, 1:1280 and 1:640, respectively.

Antisera were prepared against eight different cultures and each one of these was tested by direct agglutination with all of the eighteen cultures included in this study.

The results of the test by direct agglutination are presented in table 1.

It is seen that most of the sera were able to agglutinate some of the heterologous cultures. However, in most of these instances the cross agglutination was so slight (1:320 or less) that the question of a possible identity of the respective cultures did not need to be considered. Moreover, a later test carried out preliminary to a proposed absorption experiment, showed, in all, a considerable diminution in even this slight degree of cross agglutination, which rendered the absorption experiment entirely superfluous.

The absorption experiment was carried out with the antisera Gordon and Eldridge. The absorptions carried out with the antiserum Holmes are described below in the special study of that serum.

TABLE 1

Direct agglutinations

SERUM DILUTION	SERUM ANTI-LEE						SERUM ANTI-MASATES						SERUM ANTI-GODFREY						SERUM ANTI-MICHAEL						SERUM ANTI-GORDON						SERUM ANTI-HOLMES						SERUM ANTI-ELDRIDGE						SERUM ANTI-WILLIAMS					
	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560
Lee	+	+	+	+	±	0	±	±	X	0	0	0	0	0	0	0	0	0	±	X	0	0	0	0	0	0	0	0	0	0	±	X	0	0	0	0	X	+	0	0	0	0	±	0	0	0	0	0
Masates	0	0	0	0	±	0	±	±	+	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	0	0	0	0	0	0	0	0	0
Godfrey	0	0	0	0	0	0	+	±	0	0	0	0	+	+	+	+	0	0	+	X	X	0	0	0	X	0	0	0	0	0	+	+	+	0	0	0	±	0	0	0	0	0	±	0	0	0	0	0
Michael	Not tested						Not tested						0	0	0	0	0	0	+	+	+	0	0	0	+	+	+	+	+	±	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
Gordon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	X	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0
Holmes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	±	X	0	0	0	0	0	0	0	0	0	+	+	+	±	±	0	0	0	0	0	0	0	0	0	0	0	0	0
Eldridge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	±	X	0	0	0	+	+	+	+	±	0	+	+	+	+	+	±	+	+	+	+	±	0	+	0	0	0	0	0
Williams	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	±	±	0	0	0	0	0	0	0	0	0	0	+	+	+	0	0	0	+	0	0	0	0	0	+	+	+	+	+	0
Leuchner	+	±	+	+	±	0	+	+	±	0	0	0	+	0	0	0	0	0	+	+	±	0	0	0	+	+	+	+	+	X	+	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0
Amert	±	0	0	0	0	0	±	0	0	0	0	0	0	0	0	0	0	0	±	±	±	0	0	0	0	0	0	0	0	0	+	+	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0
Angela	X	0	0	0	0	0	X	X	0	0	0	0	0	0	0	0	0	0	X	X	X	0	0	0	+	+	+	+	±	X	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
Meyer	0	0	0	0	0	0	X	X	±	0	0	0	0	0	0	0	0	0	±	±	±	X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
126	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
159	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Witt	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G. L. T.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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+ = complete agglutination; ± = partial agglutination; X = slight agglutination; 0 = no agglutination.

TECHNIC

The bacterial sediment used in the absorption experiment represented a twenty-four-hour "chocolate-agar" slant growth which had been planted from a previous twenty-four-hour chocolate-agar culture. The bacteria were scraped off the surface of the medium, suspended in about 10 cc. of sterile saline solution and thrown down in the centrifuge.

After decantation of the supernatant fluid there was added to the sediment a quantity of the serum to be absorbed equal to one-half of the volume of the bacterial sediment. The serum

TABLE 2

Agglutinin absorptions

SERUM		AGGLUTINATION BEFORE ABSORPTION							ABSORBED BY STRAIN	AGGLUTINATION AFTER ABSORPTION											
										Of absorbing strain						Of homologous strain					
	Strains	80	160	320	640	1280	2560	Control		80	160	320	640	1280	2560	80	160	320	640	1280	2560
Gordon...	Gordon	+	+	+	+	+	×	0	Gordon	0	0	0	0	0	0	0	0	0	0	0	0
	Amert	+	+	+	+	±	×	0	Amert	0	0	0	0	0	0	0	0	0	0	0	0
Eldridge..	Eldridge	+	+	+	+	0	0	0	Eldridge	0	0	0	0	0	0	0	0	0	0	0	0
	Holmes	+	+	+	+	±	0	0	Holmes	0	0	0	0	0	0	0	0	0	0	0	0
	Godfrey	+	+	+	+	±	0	0	Godfrey	0	0	0	0	0	0	+	+	+	±	0	0

and bacterial sediment were mixed with a quantity of sterile saline solution necessary to provide a 1:16 dilution of the serum. The mixture was placed in a water-bath at 45°C. for several hours and afterwards in the ice-box over night. The supernatant fluid was tested with suspensions of twenty-four-hour growths of the cultures under consideration.

The results of the absorptions that were carried out are shown in table 2.

It is seen that the cultures Gordon and Amert are identical, this finding confirming that of Valentine and Cooper. Cultures Eldridge and Holmes seem, by the test, to be identical, while Godfrey is different. We have carried out similar absorptions

with two sera prepared against cultures Eldridge and Holmes by Miss Valentine and Miss Cooper and with both of these sera, also, we obtained nearly complete cross absorption.

In view of the discordance between our findings and those of Valentine and Cooper with respect to these two cultures, we have immunized another rabbit with culture Holmes and we have carried out absorptions with the resulting agglutinating serum. This serum agglutinated cultures Holmes and Godfrey in a dilution of 1:1280 and it agglutinated culture Eldridge in a dilution of 1:640. After absorption with culture Godfrey the serum agglutinated both cultures Holmes and Eldridge but not Godfrey. After absorption with culture Eldridge the serum agglutinated culture Holmes in a dilution of 1:1280, but could not agglutinate either culture Eldridge or culture Godfrey.

We are unable to explain these discordant results in the different absorption experiments. However, the discordance is immaterial to the main argument since the individuals from whom the cultures were obtained had been in personal contact with each other.

The foregoing study confirms the findings of Valentine and Cooper and supports the conclusion of Park that the bacillus of Pfeiffer can not be the cause of influenza.

The non-identity of different cultures of Pfeiffer's bacillus has been recognized elsewhere. Beiling found identity in two strains out of six, all of undescribed source. A paper has just been published by H. H. Bell (1a) in which this author, in a study of a large number of cultures obtained from cases of influenza in the St. Louis Children's Hospital, completely confirms the findings of Valentine and Cooper.

Neither of these investigators seems to have realized the significance of these findings with regard to the question of the etiology of influenza. Bieling indicates his belief in the etiological relationship of the bacillus of Pfeiffer to influenza by adopting the suggestion of Novacovic and of Neufeld and Papamarku that a polyvalent antigen of B. influenzae be used for the diagnosis of influenza (Widal technic).

II. AN UNUSUAL OBSERVATION UPON THE NATURAL INHIBI-
TION IN AGGLUTINATING SERUM

In table 1 it is seen that the serum obtained after immunization with culture Holmes was able to agglutinate both cultures Angela and Godfrey in a dilution of 1:1280, but it could not clump its homologous culture in any concentration within the usual limits. This phenomenon has not been hitherto observed, or at least recognized, perhaps on account of the fact that the preliminary examination of an antibacterial serum is usually carried out with only the homologous culture.

In order to exclude the possibility of a technical error, we tested the serum again by direct agglutination with the cultures Holmes, Godfrey and Angela and at the same time we assured ourselves of the identity and agglutinability of our Holmes culture by test-ing it with two anti-Holmes sera prepared by Miss Valentine and Miss Cooper. The results of this retest were identical with those obtained at the first examination.

Two possible explanations of the phenomenon presented them-selves: Either no major agglutinins had been produced in the injected rabbit or the agglutinins which had been formed against the injected culture were prevented from exhibiting their usual effect on account of the action of an inhibiting mechanism. The former possibility could not be immediately investigated; the latter was subjected at once to experiment.

The tests already described gave evidence that if the lacking agglutination of the homologous culture was due to an inhibiting mechanism, the inhibition was specific, since it did not interfere with the group agglutinations. The actual demonstration of the inhibiting mechanism was made in an experiment the results of which are presented in table 3.

In two series of test tubes were distributed constant quantities (two completely agglutinating doses) of two different anti-Holmes sera (Valentine and Cooper). To each tube of the two series were added the usual quantity of a suspension of a twenty-four-hour blood-agar Holmes culture and diminishing quantities of our anti-Holmes serum f. In both series agglutination was completely inhibited by $\frac{1}{320}$ cc. of the serum f.

A further test of the specificity of this inhibition was made by mixing the serum f with two completely agglutinating doses of the anti-sera Gordon, Masates and Michaels; these doses were respectively $\frac{1}{1280}$, $\frac{1}{320}$ and $\frac{1}{320}$ cc. In no case was agglutination in the least inhibited by as much as 0.1 cc. of undiluted serum f, or by any smaller quantity down to $\frac{1}{320}$ cc.

The existence of an inhibiting mechanism was thus demonstrated and it seemed probable, therefore, that there were major

TABLE 3

Showing the inhibiting property of serum f

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Series 1								
Serum 1108 diluted 1:16................	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Serum f undiluted....	$\frac{1}{10}$	$\frac{1}{10}$	$\frac{1}{100}$	$\frac{1}{100}$	$\frac{1}{1000}$	$\frac{1}{10000}$	$\frac{1}{100000}$	$\frac{1}{1000000}$
Suspension of Holmes culture............	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Saline solution.......	Up to a total volume of 1 cc.							
Results after twenty-four hours at 45°C..	0	0	0	0	?	+	+	+
Series 2								
Serum 725 diluted 1:16	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Serum f undiluted....	$\frac{1}{10}$	$\frac{1}{10}$	$\frac{1}{100}$	$\frac{1}{100}$	$\frac{1}{1000}$	$\frac{1}{10000}$	$\frac{1}{100000}$	$\frac{1}{1000000}$
Suspension of Holmes culture............	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Saline solution.......	Up to a total volume of 1 cc.							
Results after two hours at 45°C......	0	0	0	0	+	+	+	+

agglutinins in the serum the action of which was prevented. That this is true, was proven by the fact that upon several weeks standing the serum f rather abruptly acquired the ability to agglutinate the homologous culture. In table 4 are shown the results of the agglutination tests that were carried out on the 37th, 40th and 50th days after the serum had been obtained from the rabbit.

Two possibilities presented themselves in explanation of the inhibition. Either the agglutinins were prevented from uniting

with the homologous bacteria or after such union had taken place the usual clumping was inhibited. This question was investigated with the technic of absorption.

With 0.25 cc. of the bacterial sediment of a twenty-four hour blood agar growth of the Holmes culture were mixed 0.1 cc. of the serum f and 1.4 cc. of sterile 1 per cent saline solution. After this mixture had stood at 45°C. for four hours and in the ice-box overnight the supernatant fluid obtained by centrifugation was tested with the homologous strain and with all of the heterologous strains which the unabsorbed serum was capable of clumping. All of these tests resulted negatively, all agglutinating power having been removed.

TABLE 4

Showing the disappearance of the inhibiting property of serum f

DAY OF TEST	DEGREE OF AGGLUTINATION PRODUCED UPON CULTURE HOLMES BY SERUM F					
	Quantity of serum f					
	$\frac{1}{10}$ cc.	$\frac{1}{20}$ cc.	$\frac{1}{40}$ cc.	$\frac{1}{80}$ cc.	$\frac{1}{160}$ cc.	$\frac{1}{320}$ cc.
37th	±	±	±	±	±	±
40th	+	++	+++	+++	+	±
50th*	++++	++++	++++	++++	++	±

* On this day the serum was found, also, to cause complete agglutination in all quantities between $\frac{1}{10}$ cc. and $\frac{1}{80}$ cc.

As the homologous agglutination by the supernatant fluid could conceivably have been prevented by the continued presence of the inhibiting mechanism a second absorption experiment was carried out and this time the supernatant fluid was examined for the presence of that mechanism. The results of this experiment are presented in table 5.

It is seen that at least 93.75 per cent of the inhibiting power of the serum had been removed by the bacterial sediment. The experiment demonstrates, therefore, that the inhibition is due to the presence of a specific substance which is absorbed by the homologous culture and prevents the clumping action of the homologous agglutinins, which also are absorbed. The experiment was carried out on the 37th day of the serum's life, at which

It seems likely that the inhibiting substance of our serum f was unique, not in its quality but in its quantity in the serum. Serum f, in other words, contained an unusually large quantity of the natural inhibiting substance of agglutinating sera.

A striking difference is manifest in our observations between this natural inhibiting substance and the artificial "agglutinoid" described by Eisenberg and Volk. This difference lies in the fact that while "agglutinoid" is formed in agglutinating serum upon standing, the inhibiting substance of serum f lost its power of inhibition completely on standing.

We are unable to say whether this instability is a property the natural inhibiting substance of all agglutinating sera. However, we can record that in one serum (Eldridge) the inhibiting power that was exhibited only by the larger quantities (0.2 cc. to 0.05 cc.) was completely lost by the 21st day.

In view of the radical difference just referred to, there seems to be no ground for looking upon the natural inhibiting substance as a modified agglutinin. That it is a specific antibody is clear. Its relation to the inhibiting mechanism of the Neisser-Wechsberg phenomenon has not been investigated.

The assumption that the inhibiting substance has a greater "avidity" than the active agglutinins seems superfluous, in view of the fact, demonstrated by Eisenberg and Volk themselves, that the previous absorption of the inhibiting substance by the bacteria does not prevent the subsequent attachment of agglutinins to the same bacteria. Our own experiments show that the inhibiting and the agglutinating antibodies are absorbed together.

SUMMARY

1. Confirming the previous work of Valentine and Cooper, a study of eighteen cultures of the bacillus of Pfeiffer isolated from cases of influenza in different localities and at different times revealed identities in the cultures only when a probability of personal contact existed.

2. On the basis of Park's argument these findings admit of only one conclusion with regard to the rôle of Pfeiffer's bacillus; namely, that that microörganism is not the cause of the disease.

3. An immune serum prepared by the injection of one strain of *B. influenzae* was found to agglutinate other strains of that bacillus, but not the one used for the immunization. This phenomenon was due to the presence of an unusual quantity of a specific inhibiting antibody.

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HYPERSENSITIVENESS: ANAPHYLAXIS AND ALLERGY

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The subject of hypersensitiveness dates from the earliest observations upon human idiosyncrasy when it was noted that certain persons reacted, with peculiar symptoms, to contact with substances, such as foods, drugs, animal emanations and pollens, which to the great majority of individuals were entirely innocuous. Considerable added interest was infused into the study of this natural hypersensitiveness of human beings by the observations of Richet, Arthus, Theobald Smith and many others upon the experimental hypersensitiveness of lower animals. One of the outstanding results of this stimulated interest was the generally accepted inclusion of all of the known phenomena of peculiar or unusual physiological reactivity under one heading—that of anaphylaxis.

In the designation of such phenomena in one group the quality of peculiar reaction has generally been the only one taken into account, notwithstanding obvious and often radical differences among some of them as to their nature and causation. At present no etiological subdivision of these various phenomena is recognized.

It is the purpose of this article briefly to present restrictive definitions of the terms in general use in the literature of the subject and to classify the known phenomena of peculiar physiological reaction in accordance with these definitions.[1]

[1] A fuller exposition of this question is contained in the article on "Hypersensitiveness" in the forthcoming Practice of Medicine edited by Dr. Frederick Tice.

It may be that the term hypersensitiveness will always be needed for use in a general sense applicable to conditions of non-specific, exaggerated or unusual reactivity on the part of a living organism. Such conditions are illustrated in the exaggerated sensitiveness to light of the eye in conjunctivitis.

There are, however, two groups of the phenomena of peculiar, physiological reactivity, which, on account of definite constant features, can be separated from the others etiologically and these, though themselves of different etiology,[2] can be associated under the heading "Hypersensitiveness." These two groups have been designated with the familiar terms "Anaphylaxis" and "Allergy."

From the determining features of these two groups has been drawn the following definition of true hypersensitiveness; namely, a condition of specific or particular reactivity, with character-istic symptoms, to the administration of or contact with any substance in a quantity which to most of the individuals of the same species is innocuous.

This definition should be amplified by the following restrictive explanation:

1. The "characteristic symptoms" are generally different in the different animal species for the same group of substances.

2. They are uniform in any one species for various substances.

3. Where the exciting agent possesses a normal physiological action; for example, the drugs, the symptoms of this action are, with few exceptions, different from those of hypersensitiveness to that agent.[3]

By the terms of the foregoing definition the tuberculin reaction of Koch and the so-called toxin hypersensitiveness are excluded from the category of true hypersensitiveness; because:

1. The symptoms of the tuberculin reaction are the same in all animal species.

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[2] Etiology refers, here, to the origin of the underlying conditions, not to the exciting agents, which are often identical in both groups.

[3] All of these facts concerning the symptoms of true hypersensitiveness point to the conclusion that the phenomena under consideration are not dependent on any special property of the exciting agent, but solely upon a peculiar ad-justment of the hypersensitive individual toward that agent.

2. The symptoms of toxin hypersensitiveness are *not* different from those of the normal physiological effect of the agent.

The phenomena of true hypersensitiveness are classified by the writer as those of anaphylaxis and those of allergy and, as it will presently appear, these terms are not used as synonyms but as mutually exclusive terms.

Anaphylaxis is a state of true hypersensitiveness that is due to the presence, in certain tissues, of specific antibodies. The symptoms of anaphylaxis are caused by the meeting of these antibodies with the respective antigen in those tissues. This definition may be elucidated by the following facts:

1. The state of anaphylaxis does not occur spontaneously but as a result of an immunological procedure (active or passive). It is experimental. The exciting agents are always antigens capable of inducing the formation of precipitating antibodies.

2. Anaphylactic hypersentiveness is not inheritable; that is, it is not transmissible to the offspring from the male parent. The hypersensitiveness that is sometimes transmitted from the mother to the offspring is always identical in both with respect to the exciting agent. In such a case the transmission of the hypersensitiveness does not occur by inheritance but by a transferal of specific antibodies from the blood of the mother to that of the offspring through the placenta.

3. An animal that is anaphylactic can always be rendered specifically and completely insensitive by a suitable (gradual) neutralization of the antibodies which are responsible for the hypersensitiveness. This procedure is known as "desensitization."

If we examine the facts concerning the causation and symptoms of human hypersensitiveness or allergy, we find full conformity with the definition of true hypersensitiveness.

1. The condition is highly specific in the sense that it is exibited in about half of the hypersensitive individuals to only one substance (Cooke and Vander Veer), sometimes indeed, to only one chemical group or element.

2. The symptoms are generally different from those of hypersensitiveness in other animal species (the coryza, the gastrointestinal symptom complex, eruptions).

3. The symptoms are the same for a great variety of different substances.

In these three characteristics the hypersensitiveness of allergy resembles that of anaphylaxis. In all other respects, however, the two conditions are diametrically dissimilar. This dissimilarity is evident in the following comparison:

1. The exciting agent in anaphylaxis is always an antigenic substance; that in allergy is often non-antigenic and cannot, therefore, induce a condition of anaphylaxis.

2. The hypersensitiveness of anaphylaxis is always induced by previous introduction of an undigested antigenic substance into the body of an experimental animal. Such a procedure has not been shown to induce allergic hypersensitiveness in human beings and the weight of evidence is overwhelmingly against such an assumption. Allergy is often exhibited immediately upon the first contact with the exciting agent.

3. The hypersensitiveness of anaphylaxis is not a heritable condition. That of allergy has been proven to be inherited, and when the mother is affected the hypersensitiveness of the offspring seems to be more often exhibited to a different substance than the one that affects the mother (Cooke and Vander Veer).

4. The phenomenon of desensitization, which never fails in anaphylaxis, is entirely wanting in allergy. It is true that a certain degree of lessened sensitiveness to *natural contact* with the exciting agent is often attained in allergy, which may be termed, *clinical insensitiveness*. In all such cases, however, the suitable administration of the exciting agent, by intracutaneous or subcutaneous injection, will demonstrate the persistence of the hypersensitiveness (Cooke).

The differences between these two conditions, which have just been mentioned, may be summarized as follows:

Anaphylaxis is an experimental, or induced, non-heritable hypersensitiveness due to the presence of specific antibodies in certain tissues.

Allergy is a natural inherited condition of hypersensitiveness, which affects only human beings and is not dependent in any way on immunological antibodies.

It is clear from this comparison that the true hypersensitiveness of human beings, which we have designated as "allergy," must rest on an etiological basis that is quite different from that of anaphylaxis.

The question naturally presents itself, cannot the hypersensitiveness of anaphylaxis be induced in human beings as well as in lower animals?

It may be pointed out, here, that not all lower animals have been rendered anaphylactic.[4] The negative experiments of Yamanouchi and also by Uhlenhuth and Haendel with apes have been confirmed with a much larger number of animals by Auer in unpublished observations.[5]

For the certain recognition of anaphylaxis in human beings it would be necessary that peculiar and characteristic symptoms develop *only* after a *reinjection* of an antigenic substance. If the same symptoms are seen to be produced often by a primary injection of the antigenic substance or, indeed, more frequently after a primary injection than after a reinjection, then such evidence could hardly be used as indicating the existence of a condition of anaphylaxis. The mere absence of effect at the first injection by no means proves that the effect of the reinjection is due to the immunological mechanism. The difficulty of recognizing the hypothetical anaphylactic symptoms following the reinjection of a true antigen must increase if, as is actually the case, the symptoms observed under such circumstances are never different from those of allergy.

It becomes necessary at this point to identify the allergic symptoms; that is, to determine what symptoms in human beings may be considered to be allergic.

On the one hand allergic conditions must conform to the general criteria of hypersensitiveness and, on the other hand, they must present some feature which will exclude them from the category of anaphylaxis.

Under the definition of hypersensitiveness laid down above, allergic symptoms may be identified by their specificity, their

[4] Only the usual criteria of outward symptoms and death are considered here.
[5] Personal communication.

unusual occurrence, their uniformity with different exciting agents and their difference from the normal physiological effect of the agent.

It is a striking feature of allergy that these requirements are met by several symptoms[6] or symptom-complexes. Thus, coryza bronchial asthma, gastro-intestinal disturbance, multiform cutaneous eruptions and sudden death following dyspnoea and respiratory failure, are all seen, in some individuals, to result from natural contact with or the administration of substances that are, to most individuals, entirely innocuous.

All of these symptoms are found to occur under circumstances that exclude the possibility of an anaphylactic etiology. Such circumstances are:

1. The non-antigenic nature of the exciting agent as in drug allergy ("idiosyncrasy").

2. The development of the symptoms at the first contact with the agent, as in many cases of "serum sickness" without incubation period.

3. The demonstration of the factor of heredity in the causation of the symptoms.

Having identified the various symptoms of allergy we may turn again to the question whether anaphylactic hypersensitiveness does actually occur in man. We can consider this question most conveniently by examining the effects in human beings of the injection of therapeutic sera; because the material injected is a known and commonly employed anaphylactogen and because the conditions under which such sera are administered often approximate those of the anaphylaxis experiment.

The injection of therapeutic serum, which is practically always derived from the horse, is often followed by a reaction known as "serum disease." The symptoms of this condition are those of allergy and they include the multiform eruptions, fever, edema,

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[6]It is possible that there are other clinical manifestations of allergy that have not yet been recognized as such.

The character of the symptoms in any particular individual is determined in part by the mode of contact with the exciting agent. For example, the exciting agent of animal emanations if inhaled may cause coryza; if injected under the skin it may cause sudden death.

joint pains and sudden death. All of these symptoms and some other minor manifestations have been seen to follow a *primary* injection of the serum; that is, under a circumstance which, alone, would seem sufficient to rule out an anaphylactic (antibody-antigen) mechanism. Indeed the fact should be emphasized that the vast majority of the instances of serum disease occur after a primary injection.

Additional evidence of the non-anaphylactic nature of the "serum disease" that results from a primary injection of serum is found in the constant absence of the phenomenon of desensitization in that condition.

It is important, here, to note the effect of a reinjection of serum upon the course of serum disease in individuals that presented that condition at the first injection. Under these circumstances the symptoms following the reinjection *do not differ in character* from those that were produced by the first administration of the serum. However, there is no doubt that the period of incubation is generally shorter at the reinjection and that the severity of the symptoms is often increased. It must be emphasized that no evidence exists to indicate that this difference between the reactions occuring at a first injection and at a reinjection depends on any immunological influence.

There are left to be considered the relatively few instances in which, as in the anaphylaxis experiment in the lower animals, symptoms resulted in human beings from the reinjection but not from the first injection. These circumstances are analogous to those of active anaphylaxis and if allergy did not exist they could be accepted as representing anaphylaxis in man. However, in none of these instances have symptoms been noted which were characteristically different from those of allergy. They have been either the usual eruptive manifestations or collapse followed, in some cases, by death.

Hence, the only reason for suspecting these cases to be instances of anaphylaxis and not of allergy is the absence of symptoms at the first administration of the serum, and this circumstance cannot be satisfactorily explained until the mechanism of allergy is known. Certainly, it is not sufficient basis for a separation of these instances from the category of allergy.

In this connection it is well to bear in mind the instances in which the symptoms of drug allergy were absent at the first administration of the drug but appeared after a repeated administration. Only very few such instances have been reported, probably because many of those who have observed them have overlooked their theoretical interest.

I am permitted by Dr. John A. Fordyce to refer, in advance of their publication, to some instances of this kind that have occurred in his experience. In as many as ten or twelve cases no symptoms were caused by the first four or five intravenous injections of salvarsan. The fifth or sixth injection was followed by allergic symptoms (eruption) which recurred at all subsequent administrations, although sometimes long intervals of time (one to three years) elapsed between successive injections. In a personal communication, Dr. Fordyce remarks:

The amount of the drug which produces the relapsing eruption apparently has no special significance. The amount of drug injected after these intervals [one to three years] has usually been a minimum quantity, about 0.25 to 0.3 of a gram of salvarsan.

An exactly similar case has been observed in the dermatological clinic in Cornell University Medical College, which will be reported by Dr. Oscar L. Levin.

Thus, it is evident that the mere absence of symptoms upon a first injection of serum is not sufficient to indicate that the symptoms occurring upon subsequent injection are of anaphylactic origin; there was previous evidence, which is now confirmed by the more numerous observations of Fordyce and that of Levin, that this occurrence is a characteristic phenomenon of drug allergy—a condition obviously unrelated to anaphylaxis.

Even in the few cases, therefore, in which the possibility of the operation of an anaphylactic mechanism could be considered; that is, those in which symptoms developed only upon a reinjection of serum, there appears to be no good ground for looking upon these as anything else than less usual forms of allergy.

As is well known, the proof that the manifestations of anaphylaxis are due to an antibody-antigen reaction was provided in Otto's demonstration of passive sensitization.

The principle embodied in the experiment of passive sensitization has been applied in serum and food allergies and even in non-antigenic drug allergy and the ultimate purpose of such applications of the principle has been to prove the anaphylactic nature of those allergies. However, the purpose of such experiments has been nullified by a fallacy which seems to have been overlooked. The technic of passive sensitization can be used to detect the presence of precipitin in an individual's blood, but if the presence of precipitin has been demonstrated by this means it does not follow that the individual under examination is anaphylactic. For example: guinea-pigs have been passively sensitized with serum from an immunized monkey, yet in not a single instance in numerous experiments has the monkey itself been rendered anaphylactic.

In applying the technic of passive sensitization in the study of allergy, the investigators have transferred the serum of the human individual almost exclusively to the guinea-pig, in which, then sensitization to the exciting agent of the allergy was looked for. However, in one instance reported by Ramirez,[7] a quantity of blood (600 cc.) was transfused from an individual that was hypersensitive to horse dander to an anaemic patient who had previously exhibited no symptoms of allergy. Two weeks after the transfusion the recipient went for a carriage drive and was seized at once with an attack of asthma. The usual cutaneous test revealed in the patient a hypersensitiveness to horse dander. Unfortunately, it is not known whether the cutaneous hypersensitiveness existed previous to the transfusion or not.

This observation is unique in the records. If it should be found that under similar circumstances hypersensitiveness could be regularly or often transferred from one human individual to another, it would be necessary to revise the conception of allergy that is presented in the foregoing pages. For the present, it seems proper to look upon the occurrence reported by Ramirez as an accidental coincidence. Some support is given to this view by the fact, which appears in the paper of Ramirez, that

[7] Journ. A. M. A., 1920, 73, 984.

the same donor had supplied a larger quantity of blood (800 cc.) to another recipient, who, however, did not develop hypersensitiveness to horse.

The age of onset of the clinical manifestations of allergy is different in different individuals. Cooke and Vander Veer have shown that the age of onset depends entirely upon a hereditary influence. It seems more probable that the recipient observed by Ramirez had just reached the age of natural onset of the horse allergy when the transfusion was carried out, than that the transfusion itself was the cause of the allergy.

In view of the facts that form the basis of the foregoing discussion, it seems necessary to conclude: first, that if anaphylaxis does occur in man, it does so only very rarely and secondly, that there is no positive evidence that anaphylaxis occurs at all in human beings.

THE MECHANISM OF THE ANAPHYLAXIS REACTION IN THE RABBIT[1]

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The previous source of information regarding the pathology of anaphylaxis in the rabbit has been chiefly the publications of Auer. In a preliminary report (1), Auer referred the fatal acute anaphylactic shock in the rabbit to a direct effect upon the heart, which he designated as an "inhibition" or a "paralysis," and he demonstrated the local site of the anaphylaxis reaction in that animal by excluding central nervous and splanchnic influences before the reinjection. The changes in the heart described by Auer in this and later publications (2) were as follows.

The heart is filled with blood and shows either no ventricular beat or only a weak beat. The auricles beat in some experiments slowly and regularly. The ventricles respond weakly or not at all to mechanical or electrical stimulus.

There is a remarkable difference between the two ventricles; the left ventricular wall appears to be practically normal as to its consistence, whereas the right ventricular wall feels stiffer and less yielding than normal.

In a study (3) of the cause of death in rabbits resulting from the intravenous reinjection of foreign blood corpuscles, the writer came to the conclusion that the fatal result here was due to a mechanical obstruction of the pulmonary circulation by the agglutinated corpuscles. This conclusion was drawn from the fact that the corpuscles could regularly be found in the pulmo-

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nany capillaries and arterioles after death resulting from the
reinjection of nucleated corpuscles and from the fact that in
one instance when the corpuscles were laked before they were
used for the reinjection no symptoms followed that injection.

Since it seemed not impossible that the changes observed by
Auer could be secondary to an interruption of the pulmonary
circulation and since the histological findings in the lungs of
rabbits killed by the reinjection of corpuscles had not been
explained the investigation was resumed in the present study.

The first purpose of the investigation was to test directly the
permeability of the pulmonary circulatory system of rabbits that
had been killed with a reinjection of blood corpuscles.

Upon the advice of Dr. Robert A. Hatcher, with whom a
number of the experiments were carried out, the permeability
of the pulmonary vessels was tested as follows:

After the death of the animal the chest was widely opened and both
ventricles of the heart were cut off with a scissors; a glass cannula,
connected by rubber tubing with a glass bulb containing physiological
saline solution was inserted into the pulmonary artery through the
right ventricle and tied fast; care was taken not to include the pulmo-
nary vein in the ligature; after the bulb had been placed at the level of
the chest, the pinch-cock on the rubber tubing was opened and the
bulb was slowly raised until fluid began to flow from the left auricle;
at this point the height of the column of saline solution, measured from
the level of the heart, was noted.

In two experiments on a normal rabbit and a normal cat,
both killed with ether, a pressure of 10 cm. of salt solution was
found to produce a steady flow from the left auricle. This
result is in agreement with the estimations of the normal blood
pressure in the pulmonary artery of rabbits that were reported
by Knoll (4) and by Mellin (5) in two considerable series of
determinations. The average pressure, according to these au-
thors, is, repectively, 13 and 14.8 mm. of mercury. The maximal
pressure recorded by Knoll was 18 mm. of mercury. In further
tests with normal rabbits and guinea-pigs it was found that fre-
quently an initial pressure of as much as 30 cm. of water was

required to inaugurate the flow of the perfusion fluid through the lungs. This was sometimes seen to be due to a twisting of the pulmonary artery just beyond the orifice of the cannula or to a closure of the orifice by the wall of the vessel being stretched over it. This circumstance obviously prevents an accurate measurement of the lesser degrees of any abnormal resistance in the pulmonary circulation.

A preliminary test of the general question under consideration was made in the following two experiments.

Rabbit 133 received a primary subcutaneous injection of 3 cc. of the once washed corpuscular sediment of oxalated sheep's blood and a second injection, 8 days later, of 2.5 cc. of the same material, this time by the intravenous route. Immediately after the death of the animal, which occurred 4 minutes after the intravenous injection, the permeability of the pulmonary vessels was tested. A pressure of 49 cm. of water caused a slight ooze of fluid from the left auricle and a pressure of 70 cm. produced a somewhat increased flow, which, however, was less than the normal flow at 10 cm.

Rabbit 303–304 received a primary intravenous injection of the washed sediment of 2 cc. of chicken's blood and, 16 days later, a similar injection of 5 cc. of the same material. Death followed the reinjection in two minutes and the pulmonary circulation was found to be impermeable to physiological saline solution under a pressure below 70 cm. of water. At 71 cm. a slight ooze of fluid from the left auricle began, which increased to a slow flow at 84 cm.

In both of these experiments, as the bulb containing the saline solution was raised in testing the permeability of the pulmonary vessels, the increasing pressure was evidenced by a ballooning of the pulmonary artery about the orifice of the cannula. When the border of the lung was incised, while the pulmonary artery was under the maximal pressure of the perfusion fluid, no oozing of fluid could be detected. A deeper incision severed the larger branches of the artery from which, then, a stream of the fluid issued freely.

These experiments demonstrate the occurrence of an obstruction to the pulmonary circulation in rabbits dying after an intravenous reinjection of blood corpuscles and they seemed

to uphold the earlier belief of the writer that the cause of that obstruction was a process of agglutination.

In support of that belief it was pointed out in the earlier publication that the serum of normal rabbits contains no agglutinins for chicken's corpuscles, which are not "toxic" on a first injection, whereas such serum does contain agglutinins for pig's corpuscles, which often kill rabbits at the first intravenous injection.

The two succeeding experiments were undertaken to determine whether death following the primary intravenous injection of "toxic" corpuscles may also be due to an obstruction of the pulmonary circulation.

Normal rabbit 408–413 received, by intravenous injection, the thrice washed corpuscles of 6 cc. of pigs blood. Death followed in four minutes and the pulmonary vessels were found to be impermeable to normal saline solution under a pressure of 90 cm. At 94 cm. a slight ooze of fluid began from the left auricle.

Normal rabbit 129–169 received a similar injection of 5 cc. of pig's corpuscles. In this instance the animal fell over two minutes after the injection but did not die till fifteen minutes later. A pressure of 29 cm. caused a slight ooze of fluid from the left auricle, which, as the bulb was raised to a height of 70 cm., increased to about the rate of flow through the normal lung at a pressure of 10 cm.

These two experiments, while apparently adding further support to the agglutination theory, actually threw some doubt upon that assumption because a previous examination had shown that the serum of rabbit 408–413 contained no demonstrable normal agglutinins for pig's corpuscles. The succeeding experiments rendered the theory untenable.

The corpuscular sediment of pig's blood, which had been washed three times with physiological saline solution, was mixed with an equal volume of distilled water and after five minutes, complete hemolysis having taken place, the mixture was centrifugalized at low speed for three minutes. The resulting supernatant fluid was injected intravenously into three normal rabbits as follows:

Rabbits 2–14 and 167–170 received respectively 8 cc. and 2.5 cc. of the supernatant fluid and both died four minutes later; the pulmonary vessels in both animals were found to be entirely impermeable to physiological saline solution under a pressure of 94 cm.; rabbit 414–415 received 8 cc. of the supernatant fluid without exhibiting any symptoms.

In only one of these three animals were normal agglutinins to pig's corpuscles demonstrable; 0.2 cc. of the serum of this animal (number 2–14) produced very slight clumping of 0.5 cc. of a 5 per cent suspension of pig's corpuscles. Normal anti-pig hemolysin was present in the sera of 2–14 and 167–170 and absent in 414–415; 0.2 cc. of the fresh active serum of the last mentioned animal caused no hemolysis of 0.5 cc. of 5 per cent suspension of pig's corpuscles.

The production of an impermeable condition of the pulmonary vessels by the injection of the corpuscular substances in solution excluded the possibility here of an agglutination phenomenon as the cause of the obstruction; the observation rendered such an explanation improbable, also, in the case of the pulmonary circulatory obstruction following the reinjection of primarily non-toxic corpuscles and it suggested that the symptoms of serum or other protein[2] anaphylaxis in the rabbit are likewise due to obstruction of the pulmonary circulation.

The mechanism of the acute shock of anaphylaxis in the rabbit was studied in 4 animals—rabbits number 405–406, 407–408, 170–304, and 409–410—which had received 13 injections of egg-white as follows: 1st day, 0.5 cc.; 6th day, 1 cc.; 8th, 9th, 10th, 11th, 13th, 14th, 15th, 16th, 17th, 18th and 20th days, 0.25 cc., usually by the intraperitoneal route. The test injections were made on the twenty-fourth day.

Rabbit 405–406 received 2.5 cc. of undiluted egg-white by intravenous injection and died two minutes later. At a pressure of 76 cm. of water a slight flow of the perfusion fluid was produced through the pulmonary vessels.

[2] A distinction between "corpuscle anaphylaxis" and "protein anaphylaxis" is made here only for convenience of discussion. It will be presently seen that, as in the guinea-pig, the mechanism of the anaphylaxis reaction in the rabbit is doubtless the same whether the anaphylactogen is formed or not.

Rabbit 407–408 was treated exactly as the preceding animal and it died within two minutes after the injection. The pulmonary vessels of this animal resisted a water pressure of 86 cm.; not the least fluid was forced through the vessels under this pressure.

The foregoing experiments demonstrate that in anaphylaxis in the rabbit the acute shock is accompanied by an obstruction to the pulmonary circulation, which, in both of the animals tested, may be considered physiologically complete.

As to the mechanism of this obstruction two possibilities suggested themselves. One of these was that a specific precipitate might be formed upon the reinjection of the protein at the test and that this precipitate could cause an embolic closure of the pulmonary capillaries and arterioles.

The possibility of a mechanical interference with the circulation by specific precipitates, formed *in vivo*, was considered in relation to the symptoms of serum allergy by Rostoski (6) and by Michaelis and Oppenheimer (7) but all of these authors rejected the theory on the ground that in such a case death must result and they concluded, from the fact that death did not follow the injection of a foreign serum into rabbits, even in the presence of demonstrable precipitin, that specific precipitates are not formed *in vivo*. The argument on which this conclusion rests is evidently invalidated in anaphylactic shock in the rabbit, in which case death does follow the injection.

In investigating the possibility under consideration the process of perfusion was resorted to with the purpose of seeing whether a removal of the greater part of the blood contained in the lungs would prevent the development of the obstruction to the pulmonary circulation when the antigen was then introduced into it.

Rabbits 170–304 and 409–410 (for previous treatment see page 222) were killed with a blow on the neck and a cannula was fastened in the pulmonary artery. With the bulb at a height of 33 cm. and 28 cm. respectively, a rapid flow of warm physiological saline solution was maintained through the pulmonary vessels for 5 minutes. The flow was then interrupted with a pinch-cock and 2.0 cc. of undiluted egg-white were injected into the lumen of the rubber tubing between the

pinch-cock and the cannula. One minute later the pinch-cock was loosened and the bulb was slowly raised. In one case (170–304) a pressure of 77 cm. of water caused a slight ooze of fluid from the left auricle; in the other (409–410) not the least fluid could be forced through the lungs under a pressure of 92 cm. of the perfusion fluid.

A certain limitation of the procedure just described has been pointed out by Larson and Bell (8), who showed that the perfusion of isolated organs with physiological saline solution does not guarantee the complete removal of the blood that is present in the organ at death, since considerable areas are not reached by the perfusion fluid. This limitation[3] cannot be disregarded. It prevents the acceptance of the foregoing results as disproving the occurrence of pulmonary embolism as a result of specific precipitate formation in the lumen of the pulmonary vessels, although these results contribute further evidence that the intravenous reinjection of an anaphylactogen in the rabbit causes a physiologically complete occlusion of the pulmonary circulation.

Further light on the question under consideration was sought with the use of toxic normal serum. It is known that in the guinea-pig and the dog, the effect, both as to symptoms and pathology, of the primary injection of toxic normal serum is indistinguishable from that of a reinjection of a non-toxic protein. This is evidently due to a peculiar susceptibility on the part of certain tissues, which are different in the two animals, (the unstriped bronchial musculature in the guinea-pig and the liver cells in the dog); a peculiar susceptibility to various agents; for example, pepton. It may be assumed, therefore, that if the injection of normal serum into the rabbit produces the symptoms and pathology of anaphylactic shock in that animal, it does so through its influence upon the mechanism that is involved in the shock of anaphylaxis.

The experiments with toxic serum (fresh unheated sheep's serum) were carried out upon two normal rabbits, x and y, of 1400 grams and

[3] It may be remarked here that while the limitation pointed out by Larson and Bell clearly applies when the perfusion is carried out on the isolated organs, it has been found (8) not to apply when the perfusion is conducted upon the living animal.

1350 grams weight, respectively. In both instances 20 cc. of the serum were injected intravenously and death occurred in 1½ minutes and 5 minutes respectively. The pulmonary vessels in rabbit x were found to be entirely impermeable to salt solution under a pressure of 82 cm., while those of rabbit y resisted a water pressure of 68 cm. (higher pressures were not applied).

In rabbit x the right ventricle and the abdominal veins were found, at autopsy, to be completely filled with clotted blood and the possibility had to be considered that in this instance it was the clotted blood which hindered the passage of fluid through the pulmonary vessels. However, this explanation was rendered unlikely by the following experiment, which was carried out on a normal guinea-pig with the sheep's serum.

Six cubic centimeters of the serum were injected intravenously into a guinea-pig of 500 grams weight; death followed in three minutes with the typical symptoms and pathology of anaphylactic shock in this animal. The right heart was found to be filled with clotted blood, exactly as in rabbit x; yet the pulmonary vessels were readily permeable to physiological saline solution under a pressure of only 10 cm.

This experiment indicates that the mere presence of recently clotted blood in the pulmonary vessels is not sufficient to prevent the passage of fluid through them.

The results of the foregoing experiments with toxic serum indicate that the occlusion of the pulmonary vessels, which is peculiar to the pathology of anaphylactic shock in the rabbit, is not brought about by an embolic process, because the normal rabbit's blood does not possess the power of precipitating sheep's serum.

We are lead, by this conclusion, to the alternative assumption that the obstructing mechanism, which is affected both by the reaction of anaphylaxis and by primarily toxic serum, is situated in the vessel wall and that mechanism must be the muscular coat of the arteries.

It is not to be supposed that it is only the pulmonary artery of the rabbit which possesses the susceptibility that we are considering, but that this peculiarity is shared by the entire arterial

system. When the test material is administered by intravenous injection it is the pulmonary artery that comes first under its influence and it is, therefore, the interruption of the pulmonary circulation that dominates the pathology of the acute shock of anaphylaxis in the rabbit. However, when the administration is by subcutaneous injection it should be expected that a corresponding obstruction will be produced in all of the local arteries and that this will result in the formation of an area of anaemic infarction. In fact such an effect of subcutaneous injection in the rabbit has long been known in the local phenomenon of Arthus.

Thus, two of the three expressions of anaphylaxis in the rabbit are referable to the same mechanism and it remains to be seen whether the third expression of anaphylaxis; namely, that of the delayed shock or cachexia of Arthus, is susceptible of a similar explanation.

Opportunity to study the pathology of the cachexia of Arthus was presented in only one instance; hence the findings in this animal, although they were unequivocal, cannot be accepted without further confirmation. The history of the animal is as follows.

Rabbit 168–187, weighing 2800 grams, received on the 2nd, 6th and 21st of September, 2 cc., 2 cc. and 1 cc. of dog's serum injected subcutaneously. On September 28 the animal received an intravenous injection of 2 cc. of dog's serum and 2 minutes later it fell over in clonic convulsions, from which, however, it soon recovered. One further injection of 4 cc. of dog's serum was given on October 3 by the intravenous route; this injection caused only a period of drowsiness interrupted by occasional starting up. Excepting the first injection the serum used had been heated for one-half hour at 55°C.

On October 23 the rabbit was found dead and the following conditions were observed at the autopsy.

The animal was markedly emaciated; it weighed 1970 grams. The muscles were pale. The pleural cavity on both sides was filled with a clear straw colored fluid and a considerable quantity of a similar fluid was present in the abdominal cavity. The right auricle and ventricle were greatly distended and filled with a mixed red and white blood clot. The right ventricular cavity measured 1 by 1.5 cm.

The right ventricular wall presented an area of about the size of a dime which was so thin as to be translucent. This condition of the wall as well as the great dilatation of the cavity of the right ventricle is shown in the photograph (fig. 1), in which two normal rabbits' hearts have been placed on either side of the heart of anaphylactic cachexia. Microscopic examination of the thinned area revealed the entire absence of muscular elements; these were replaced with fibrous tissue. As is seen in the photograph,[4] the left ventricular cavity was relatively contracted; the left auricle was in a similar condition. The valves of the heart were normal. The lungs were diminished in volume and of markedly increased consistence. The consistence of the left lung was leathery like that of atelectasis and portions of this lung sank

FIG. 1

when placed in water. There was no edema in the lungs and no gross nor microscopic evidence of inflammation. Microscopic examination showed a thickening of the interalveolar septa, which, in the left lung, had resulted in a practically complete obliteration of the alveolar cavities. The aorta was collapsed and contained a narrow ribbon of clotted blood, which occupied but a fraction of the otherwise empty lumen. The vena cava and the abdominal veins were greatly distended with blood.

[4] In the middle of the photograph are the right and left ventricles of the heart of rabbit 168-187. The light area on the inner aspect of the right ventricle marks the area of thinning, which has been cut through. The hearts at the right and left are from normal rabbits.

All of these findings are characteristic of the effect of a chronic mitral or pulmonary stenosis, an effect which obviously must be the same if an obstruction to the circulation occurs in the lung. The assumption of the latter site of obstruction is justified here since both the mitral and the pulmonary valves were entirely normal.

In a single experiment the mechanism of acute anaphylactic shock was examined in a rabbit that had been passively sensitized.

A small normal rabbit, weighing 900 grams, received, by intravenous injection, 15 cc. of the unheated serum of a rabbit that had been highly immunized against dog's serum. Five minutes later 2 cc. of inactivated dog's serum were injected intravenously. Death followed ten minutes after this injection and the pulmonary vessels were found to be impermeable to saline solution under a pressure of less than 92 cm. of water.

We are now in position to explain the observation, reported in the earlier publication (3), that in the lung of a rabbit which has been killed by a reinjection of chicken's corpuscles the arterioles and capillaries are found to be filled with the nucleated cells and apparently occluded by them. This finding is explained by the assumption that the anaphylactogenic substances are liberated from the injected corpuscles so quickly in the blood current of the rabbit that by the time the corpuscles reach the lungs the concentration of the liberated substances is sufficient to cause the occlusive contraction of the muscular coat of the pulmonary arteries. As soon as this occlusion is physiologically complete, the flow of the blood ceases and the nucleated corpuscles remain wherever they happen to be, whether their situation is proximal or distal to the point of occlusion.

This explanation is supported by the following experiment.

Two rabbits that had received repeated intraperitoneal injections of egg-white were given finally an intravenous injection of washed chicken's corpuscles suspended in egg-white. Both animals died within a few minutes after the final injection and in both instances microscopic examination of the lungs revealed the presence of numerous nucleated corpuscles in the pulmonary arterioles and capillaries.

Excepting the experiments with nucleated corpuscles, the histological study of the lungs of normal rabbits and of those that have died in acute anaphylactic shock has failed to reveal a consistent difference in the condition of the pulmonary artery which would confirm the findings obtained with the technic described above.   This study, however, will be continued.

It has been suggested above that the changes observed in the right ventricle by Auer are conceivably secondary to the interruption of the lesser circulation.   Such an occurrence must affect the right heart by withholding nourishment from it and also by increasing to a maximum the work required of the right ventricle.   The left ventricle is likewise deprived of nourishment, but as the systemic blood pressure falls quickly to almost zero, the left ventricle is soon relieved of the stimulus to work. This may explain the differences between the two ventricles described by Auer.

SUMMARY

In acute anaphylactic shock in the rabbit, whether induced with cells (corpuscles) or with dissolved protein, and after both passive and active sensitization, an occlusion of the pulmonary vessels is constantly observed.

Experiments with dissolved corpuscles and with primarily toxic serum indicate that this occlusion is not embolic but is due to a contraction of the muscular coat of the arteries comparable with that of the bronchial musculature in anaphylactic shock in the guinea-pig.

The local phenomenon of Arthus and the cachexia of the rabbit that was observed by the same author appear, also, to be the result of a similar interference with the circulation.

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STUDIES IN ANAPHYLAXIS

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I. ON THE QUANTITATIVE REACTION OF PARTIALLY NEUTRALIZED PRECIPITIN IN VITRO AND IN VIVO

In one of the numerous studies (1) with which Richard Weil enriched the literature of anaphylaxis he came to the conclusion that the reaction between antigen and antibody that occurs within or upon the susceptible cells in the anaphylactic guinea-pig takes place in a manner different from that of the reaction of these two substances *in vitro*. This conclusion was drawn from a quantitative study of the anaphylactic reactivity of passively sensitized guinea-pigs that had been partially desensitized.

Weil states that when he passively sensitized guinea-pigs with different amounts of immune rabbit's serum and partially desensitized these different animals with the same quantity of the antigen he found that the animals were all equally sensitive to a further injection of the antigen; the "minimal anaphylactic dose" of the antigen was the same for all of them. The result was the same when guinea-pigs passively sensitized with the same amount of the immune serum were partially desensitized with varying amounts of the antigen; with all of these animals, also, the "minimal anaphylactic dose" of the antigen was the same.

These experiments, which are summarized in Weil's tables 12 and 17, revealed a second peculiarity which forced Weil to the conclusion that "partially desensitized (or neutralized) antibody reacts to antigen in a manner which is quite different from that of pure antibody." This peculiarity lies in the fact that the

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minimal anaphylactic dose of the antigen was much greater for the partially desensitized animals—0.5 cc.—than it was for the animals that had been sensitized with any smaller dose of immune serum, but without being partially desensitized—0.005 to 0.05 cc. According to Weil this latter phenomenon made it seem evident "that desensitization cannot be explained on the basis of the neutralization or saturation of a fraction of the cellular antibody."

Weil wrote that these two phenomena have no counterpart in the precipitin reaction *in vitro* nor, indeed, "in immunological science." However, since he did not attempt to reproduce the phenomena in the test tube, we have taken up this question experimentally in the present study.

The usual plan of our investigation was, on the one hand, to sensitize guinea-pigs passively with a precipitating immune rabbit's serum and, after partial desensitization with varying amounts of the antigen, to determine the minimal fatal dose of the latter in the partially desensitized animals; on the other hand we mixed in test tubes quantities of the immune serum and antigen that corresponded with those used in these animal experiments and after removal of the resulting precipitate by centrifugation we determined the minimal precipitating amount of the antigen with the supernatant fluid. The supernatant fluid was also injected into a series of guinea-pigs and the minimal lethal dose of the antigen was determined for the animals so treated.

By a comparison of the results of these parallel tests, it can be seen whether the interaction of precipitin and precipitinogen is different *in vivo* and *in vitro* as Weil thought.

Such experiments were carried out with the pseudoglobulin[1] of horse-serum and with crystalline albumin prepared from the white of hen's egg.

The immune serum used in the first experiment was a mixture of sera derived from two rabbits (170 and 397) that had received

[1] For a generous supply of this material we are indebted to Charles R. Tyler, Research and Antitoxin Laboratory, Department of Health of the City of New York, Otisville, N. Y.

numerous intraperitoneal injections of crystallline egg albumin, as follows: 2 cc., 10 cc. and 5 cc. on the first, fifth and eleventh days, and 0.5 cc. daily from the thirteenth to the twenty-sixth days inclusive. The animals were bled four days after the last injection.

The minimal sensitizing dose[2] of this serum mixture was found to be 0.2 cc.

The minimal anaphylactic dose of the egg albumin was determined for guinea-pigs that had been sensitized twenty-four hours previously with 0.4 cc. of the serum mixture. The protocol of this determination is presented in table 1.

TABLE 1

Determination of the minimal lethal dose of egg-albumin after a sensitizing dose of 0.4 cc. of serum 170+397

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.0025	Slight symptoms*
2	0.005	Severe symptoms
3	0.0075	Moderate symptoms
4	0.01	✠
5	0.01	✠

*✠   = typical anaphylactic death within 2½ to 5 minutes.
Severe symptoms   = immediate violent convulsions with eventual recovery.
Moderate symptoms = slight to moderate convulsions usually beginning after 4 to 5 minutes.
Slight symptoms   = marked dyspnoea; animal lies down on side; no convulsions.

The minimal lethal dose of the albumin was found to be 0.01 cc. 5 cc. of the immune serum (170 and 397) were then mixed with 0.01 cc. of the crystalline egg albumin solution and after two hours at 56°C. the clear supernatant fluid, which was separated from the precipitate by centrifugation, was placed in the ice-box. On the following day, no further precipitation having taken place, the fluid was compared with the untreated serum as to its precipitin titer and as to its sensitizing function.

[2] In all of these experiments guinea-pigs weighing between 250 and 340 grams were used.

TABLE 2

Comparison of the precipitation titer and complement-fixation titer of the original and the partially neutralized serum (170 + 897). Partial neutralization in vitro: the serum and antigen (egg-albumin) were mixed well and incubated two hours and then centrifuged; the supernatant fluid was tested on the following day

PROPORTION OF THE IMMUNE SERUM AND ANTIGEN MIXED				TITER OF THE PRECIPITATION AND COMPLEMENT FIXATION (ANTISERUM 0.1 CC. IN EACH TUBE)							
Series	Serum (cc.)	Antigen (egg-albumin) (cc.)	Antigen, cc.	0.001	0.0005	0.0002	0.0001	0.00005	0.00002	0.00001	0.000005
1	Untreated		Precipitation	+++	++	++	+	+	±	–	–
			Complement-fixation	0*	0	0	0	sl.	C. H.	C. H.	C. H.
2	5.0 (0.4)	0.01 0.0008	Precipitation	++(+)	++	+	+	±	–	–	–
			Complement-fixation	0	0	0	sl.	mod.	C. H.	C. H.	C. H.
3	5.0 (0.4)	0.02 0.0016	Precipitation	++	++	+	±	–	–	–	–
			Complement-fixation	0	0	sl.	mod.	N. C.	C. H.	C. H.	C. H.
4	0.8 (0.4)	0.016 (0.008)	Precipitation	+	±	–	–	–	–	–	–
			Complement-fixation	sl.	mod.	C. H.	C. H.	C. H.	C. H.	C. H.	C. H.

*0 = no hemolysis; sl. = slight hemolysis; mod. = moderate hemolysis; C. H. = complete hemolysis.

The results of the comparative precipitin titration are presented in table 2 (series 1 and 2).

It is seen that the minimal precipitating quantity of the egg albumin was about twice as great for the partially neutralized immune serum as it was for the untreated serum.

A dose of 0.4 cc. of the supernatant fluid was injected into each of a series of guinea-pigs and on the following day the minimal lethal dose of the egg albumin solution for these animals was determined. The results of the test are presented in table 3.

TABLE 3

Determination of the minimal anaphylactic dose of egg-albumin after partial neutralization of precipitin (in vitro); 5 cc. serum (170+397)+0.01 cc. egg-albumin after two hours at 37°C. centrifuged; 0.4 cc. of the supernatant fluid is used for the sensitization of each animal

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.01	Slight symptoms
2	0.02	Severe symptoms
3	0.02	✠
4	0.02	✠
5	0.03	✠

It is seen that, in harmony with the precipitin titrations, the minimal lethal dose of the egg albumin was about twice as great for these animals as it was for guinea-pigs sensitized with the same amount of the untreated immune serum.

In another series of animals the foregoing partial neutralization of the immune serum was carried out *in vivo* in accordance with Weil's procedure.

Each animal received 0.4 cc. of the immune serum[3] and on the third day thereafter each received, by intraperitoneal injection, 0.008 cc. of the egg albumin solution. Twenty-four hours later the minimal lethal dose of the egg albumin was determined as usual for the animals.

The results of the test are shown in table 4.

[3] Throughout this study the passive sensitization was effected by intraperitoneal injection.

TABLE 4

Determination of the minimal anaphylactic dose of egg-albumin after partial desensitization of passively sensitized guinea-pigs. Sensitization: with 0.4 cc. of serum 170+397. Partial desensitization on the third day: 0.008 cc. of egg-albumin intraperitoneally. Test on the fourth day

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.04	No symptoms
2	0.1	Severe symptoms
3	0.1	Very severe symptoms
4	0.2	Very severe symptoms
5	0.2	✠

It is seen that the lethal dose of the antigen by the partial desensitization method of Weil is relatively the same as it is after the corresponding partial neutralization *in vitro*.

In a further series of animals the procedure was the same as that of the preceding partial desensitization experiment except that 0.8 cc. instead of 0.4 cc. of the immune serum were used for the passive sensitization.

The results of the test in this series are shown in table 5.

TABLE 5

Determination of the minimal anaphylactic dose of egg-albumin after partial desensitization of passively sensitized guinea-pigs. Sensitization: with 0.8 cc. of serum 170+397. Desensitization on the third day: 0.008 cc. of egg-albumin intraperitoneally. Test on the fourth day

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.1	Moderate symptoms
2	0.1	Moderate symptoms
3	0.1	✠
4	0.1	✠
5	0.2	✠

It is seen that the animals used in the two tests presented in tables 4 and 5 responded too irregularly to allow a satisfactory comparison of the results in the two series although, on the whole, the animals of table 5 appear to have been more sensitive than those of table 4.

For the reader's convenience the results of the foregoing ana-
phylaxis experiments are summarized in table 6.

TABLE 6

Summary of the foregoing experiments

SENSITIZING DOSE SERUM 170 + 397	NEUTRALIZING DOSE EGG-ALBUMIN		MINIMAL ANAPHYLACTIC DOSE OF EGG-ALBUMIN
	In vitro	In vivo	
cc.	cc.	cc.	cc.
0.4			0.01
0.4	0.0008		0.02–0.03
0.4		0.008	0.2 –0.3
0.8		0.008	0.1 –0.2

In table 2 are summarized the parallel experiments in partial
neutralization of the precipitin *in vitro*. The results of these
latter tests show that the minimal precipitating quantity of the
antigen increases exactly in proportion to the amount of the
antigen which is used in the partial neutralization.

The animal and test-tube experiments taken together indicate
that, whether the partial neutralization is carried out in the test
tube or in the guinea-pigs, the quantitative relationship between
antigen and partly neutralized precipitin is the same *in vivo* and
in vitro.

A second series of experiments was carried out with the
pooled sera of two rabbits (425 and 426), both of which had
received three intraperitoneal injections of whole egg white as
follows: On the first day 5 cc.; on the fifth and tenth days 10 cc.
each time. The rabbits were bled on the seventeenth day. In
a preliminary test 0.1 cc. of each of these sera had been found
capable of fully sensitizing a guinea-pig of about 325 grams
weight. Smaller amounts of the sera were not tested.

As in the previous experiments we first determined the mini-
mal anaphylactic dose of the crystalline egg albumin solution
for guinea-pigs highly sensitized with the immune rabbits'
serum; that is, with 0.3 cc. of the pooled serum 425 and 426.
The results of this determination are presented in table 7.

The minimal anaphylactic dose is seen to have been a little more than 0.002 cc. of our crystalline albumin solution.

Three further series of animals were sensitized each with 0.3 cc. of the serum 425 and 426 and, after a partial desensitization with the egg albumin solution in amounts differing in the three series, the minimal anaphylactic dose was determined. The results of this experiment are shown in table 8-a, b and c.

Here again the minimal anaphylactic dose of the antigen increases in exact proportion to the increase in the amount of the antigen used for the partial desensitization (0.00025 : 0.0025 :: 0.035 : 0.35).

TABLE 7

Determination of the minimal anaphylactic dose of antigen after passive sensitization with the untreated immune rabbits' serum. Sensitization: with 0.3 cc. of the pooled serum (425 + 426)

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.00075	No symptoms
2	0.0015	Mild symptoms
3	0.002	Severe symptoms
4	0.002	✠
5	0.005	✠

It is seen that the injection of 0.01 cc. of our solution of crystalline egg albumin completely desensitized the animals. This result is in quantitative disagreement with those of Weil.[4] For although Weil's animals were sensitized with approximately the same amount of precipitating serum as ours (0.2 to 0.4 cc.) and although they were equally sensitive to the antigen (0.001 cc. of Weil's 5 per cent solution as compared with 0.002 cc. of our 3 per cent solution) they were only partially desensitized with 0.01 cc. and 0.04 cc. of the 5 per cent solution of the antigen. Our five animals were all completely desensitized with 0.01 cc. of a 3 per cent solution of the antigen.

With the serum mixture 425–426 partial neutralization *in vitro* was carried out exactly as in the experiments with serum mixture 170–397.

[4] See Weil's table 18.

The results of this experiment are presented in table 9.

It appears from these tests that after the immune serum 425–426 had been partially neutralized with 0.0025 cc. of the antigen the minimal precipitating dose of the antigen was about ten times as great as it was after a partial neutralization with

TABLE 8

Determination of the minimal anaphylactic dose of antigen after partial desensitization (in vivo)

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT

a. Sensitization: with 0.3 cc. of serum (425+426). Partial desensitization on the third day with crystalline egg-albumin, 0.00025 cc. intraperitoneally.

	cc.	
1	0.0133	Slight symptoms
2	0.02	Severe symptoms
3	0.025	Slight symptoms
4	0.03	Severe symptoms
5	0.035	✠

b. Sensitization: with 0.3 cc. of serum (425+426). Partial desensitization on the third day with crystalline egg-albumin, 0.0025 cc. intraperitoneally.

1	0.2	Slight symptoms
2	0.3	Mild symptoms
3	0.3	✠
4	0.35	✠
5	0.35	✠

c. Sensitization: with 0.3 cc. of serum (425+426). Desensitization on the third day with crystalline egg-albumin; 0.01 cc. intraperitoneally.

1	1.0	No symptoms
2	1.0	No symptoms
3	2.0	No symptoms
4	2.0	No symptoms
5	2.0	No symptoms

0.00025 cc. of the antigen. Corresponding with the animal experiment, 0.01 cc. of the antigen completely neutralized the precipitin in 0.3 cc. of the immune serum.

The results of the parallel tests with the method of complement fixation were in satisfactory agreement with those of the precipitin tests.

TABLE 9

Comparison of the precipitation titer and complement-fixation titer of the original and the partially neutralized serum (425+426). Partial neutralization same as before

PROPORTION OF THE IMMUNE SERUM AND ANTIGEN MIXED			TITER OF THE PRECIPITATION AND COMPLEMENT-FIXATION															
Serum cc.	Antigen (egg-albumin) 3 per cent cc.	Antigen........	0.05	0.02	0.01	0.005	0.002	0.001	0.0005	0.0002	0.0001	0.00005	0.00002	0.00001	0.000005	0.000002	0.000001
Un-treated		Precipitation	−							+++	++	++	+(+)	+	±	±	−
		Complement-fixation								0	0	0	0	mod.	C	C	C
0.3	0.00025	Precipitation								++	+(+)	+	±	±	−	−	−
		Complement-fixation								0	0	mod.	mod.	C	C	C	C
0.3	0.0025	Precipitation		+	+(+)	+++	+++(+)	+++	+++(+)	++	−	−	−	−	−	−	−
		Complement-fixation		−	−	0	0	0	0 weak	C	C	C	C	C	C	C	C
0.3	0.1	Precipitation	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Complement-fixation	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C

One quantitative discrepancy is apparent between the results of the animal experiment and those of the test tube experiment. The ratio between the normal minimal anaphylactic dose of the antigen (0.002 cc.) and that after partial desensitization with 0.00025 cc. of the egg albumin solution (0.035 cc.) is not the same as the ratio between the normal minimal precipitating dose of the antigen (0.000002 cc.) and that after partial neutralization *in vitro* with 0.00025 cc. (0.00001 cc.).

This discrepancy could be largely explained by a retest of the immune serum on the seventh day after it had been obtained from the rabbits. At this retest it was found that the minimal anaphylactic dose of the antigen for guinea-pigs passively sensitized with 0.3 cc. of the immune serum had increased to at least 0.005 cc.[5] A retest of immune serum 170–397 after it had been kept for one month in the ice-box showed a similar increase in the minimal anaphylactic dose of the antigen; that is from 0.01 cc. to 0.02 cc.

As the desensitization experiments had been carried out on the fifth or sixth days after the serum had been drawn, it is likely that the minimal anaphylactic dose of antigen had already increased.

The experiments with horse serum pseudoglobulin were carried out with the pooled sera of two rabbits (322 and 388) which had received two intraperitoneal injections of pseudoglobulin as follows: first day 1 cc.; fifth day 5 cc. The rabbits were bled seven days after the second injection.

The minimal sensitizing dose of the pooled sera was found to be 0.8 cc. The minimal anaphylactic dose of the antigen for guinea-pigs that had been sensitized with four doses of the pooled immune sera 322–388 was found, as is seen in table 10, to be 0.02 cc.

In two series of animals we studied the effect of partial neutralization of the precipitin on the minimal anaphylactic dose of antigen. In the first series of animals the partial neutralization was carried out in the test tube and the supernatant fluid ob-

[5] 0.004 cc. produced only slight symptoms; 0.005 cc. killed the only animal tested.

tained after the partial precipitation was tested with the ana-
phylaxis reaction and also with the precipitin reaction and the
complement fixation method *in vitro*.

TABLE 10

*Determination of the minimal anaphylactic dose of pseudoglobulin after passive
sensitization. Sensitization: with 3.2 cc. of serum 322+388.*

GUINEA-PIG	PSEUDOGLOBULIN OF HORSE SERUM INTRAVENOUSLY INJECTED	RESULT
	cc.	
1	0.01	Very severe symptoms
2	0.015	Very severe symptoms
3	0.02	• ✠

The results of these parallel tests are presented in tables 11
and 13.

TABLE 11

*Determination of the minimal anaphylactic dose of antigen after partial neutrali-
zation of the precipitin (in vitro). 18 cc. serum (322+388)+0.4 cc. pseudoglobu-
lin (1-56). After two hours in the incubator and twenty hours at room tem-
perature, the mixture was centrifuged and the supernatant fluid was used for
sensitization. 3.2 cc. of the fluid was injected intraperitoneally into each animal.
The tests were made on the following day*

GUINEA-PIG	PSEUDOGLOBULIN OF HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.05	Very slight symptoms
2	0.1	Severe symptoms
3	0.1	Death (delayed shock)
4	0.15	✠
5	0.15	✠

TABLE 12

*Determination of the minimal anaphylactic dose after partial desensitization. Sen-
sitization: with 3.2 cc. of serum 322+388. Partial desensitization: on the third
day with 0.284 cc. of pseudoglobulin (1-112), intraperitoneally.*

GUINEA-PIG	PSEUDOGLOBULIN OF HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.2	Moderate symptoms
2	0.3	Moderate symptoms
3	0.3	Moderate symptoms
4	0.4	Very severe symptoms
5	0.4	✠

TABLE 18

Comparison of the precipitation titer of the original and the partially neutralized serum (322+388). Partial neutralization in vitro: serum and antigen (pseudoglobulin of horse serum) are mixed well and incubated 4 hours and then left twenty hours in room temperature. The mixture is centrifuged and the supernatant fluid is tested

PROPORTION OF THE IMMUNE SERUM AND ANTIGEN MIXED			TITER OF THE PRECIPITATION (ANTISERUM 0.1 CC. IN EACH TUBE)																
Serum	Antigen (pseudoglobulin)	Antigen	0.2	0.1	0.05	0.02	0.01	0.005	0.002	0.001	0.0005	0.0002	0.0001	0.00005	0.00002	0.00001	0.000005	0.000002	0.000001
(1) 18 cc. (1–56) (3.2)	0.4 cc. (1–56) (0.00127)	Precipitation					+++	+++	+++	+++	++	++	+	±	±	−	−	−	−
(2) Untreated serum		Precipitation					+++	++++	++++	+++	+++	+++	+++	++	++	+	±	±	−

It is seen that the minimal anaphylactic dose of the antigen was increased by the partial neutralization about $7\frac{1}{2}$ fold, while the minimal precipitating amount of the antigen was increased apparently about 10 fold. This slight discrepancy is, no doubt, due to the inherent inaccuracy in the precipitation method.

In another series of animals which had been passively sensitized with the untreated serum mixture 322–388, the effect of partial desensitization on the minimal anaphylactic dose of the antigen was determined.

The results of this determination are presented in table 12.

The amount of antigen used for the partial desensitization was relatively twice that used for the preceding partial neutralization experiment.

It is seen that in this experiment the minimal anaphylactic dose of the antigen was somewhat more than twice that after the partial neutralization experiment. This apparent discrepancy can be explained in the same way as those previously noted. In this instance the immune serum was four days old at the time of the test-tube experiment and seven days old at the time of the desensitization experiment, at which time, therefore, the minimal anaphylactic dose of the antigen had increased for the untreated immune serum.

All of the experiments that have been described above point to the conclusion that precipitin remains unaltered quantitatively and qualitatively in the guinea-pig for several days and that it reacts in the animal body with its antigen in exactly the same manner as it does in the test tube. Furthermore, the experiments offer additional evidence pointing to the identity of precipitating and sensitizing antibody.

II. ON ANTISENSITIZATION

A condition of resistance to passive sensitization with a heterologous immune serum was observed by Richard Weil to result from the previous injection of the normal heterologous serum and this condition was designated by Weil as "antisensitization." The following facts were adduced by Weil regarding the phenomenon:

1. The previous injection of rabbit's serum obstructs the passive sensitization with rabbit's immune serum but not with a homologous (guinea-pig's) immune serum.

2. The interference is established after an incubation period, which is longer (eight days) after small injections (0.1 to 0.5 cc. repeated) than after large injections (1 to 8 cc.); that is, four days.

3. The duration of the normal period of heterologous passive sensitization in guinea-pigs is six days. If the guinea-pigs have received 0.1 cc. of normal rabbit's serum two to eight days previously, the period of passive sensitization with heterologous serum is shortened to barely five days. This period may be shortened, also, by the injection of 0.6 cc. of normal rabbit's serum made on the day following the passively sensitizing injection.

4. The refractory condition of "antisensitization" persists for at least sixty-eight days.

5. Active sensitization is unaffected by the injection of large amounts of normal rabbit's serum.

Weil believed the condition of antisensitization to be due to the interference of antibodies and he supported this belief with the demonstration of an obstructive action on the part of the serum of guinea-pigs that had been immunized with normal rabbit's serum. This view of Weil was apparently contradicted by his own observation that the resistance of antisensitization is not specific; it could be induced by the previous injection of the serum of the sheep and the dog or that of man.

Weil met this difficulty by demonstrating that guinea-pigs sensitized to rabbit's serum are actually hypersensitive to large injections of the three apparently unrelated sera; that is, to sheep's serum, dog's serum and human serum.

As it has been pointed out elsewhere (2), this observation of Weil is in disagreement with those of Ehrlich and Sachs, who reported that their so-called antiamboceptor was specific.

It may be remarked that if Weil's explanation of the apparent non-specificity of his anti-antibodies is to be accepted it must be upon the assumption that the sensitizing antibodies of the

immune rabbit's serum are identical, in their antigenic specificity, with the common partial antigens of the three non-
related sera, an astonishing coincidence.

If, as Weil believed, the phenomenon of antisensitization is
due to the action of anti-antibodies, it should be possible to
demonstrate a corresponding interference with passive sensitization when the immune serum, previous to its injection, is
mixed with an anti-immune serum. Moreover, it should be
possible to show that this interference is actually due to the
specific precipitation ("neutralization") of the sensitizing
antibodies.

With the purpose of applying this test to Weil's theory of the
mechanism of antisensitization, we have carried out the succeeding experiments. On April 2, 7 and 12, three normal guinea-
pigs received intraperitoneal injections of 0.5 cc., 1 cc. and 1
cc. respectively of the serum of a rabbit that had been highly
immunized with egg albumin. On April 18 these three animals
were bled to death and the sera obtained from the defibrinated
blood were pooled. The mixed serum was tested, as to its
precipitating power, with rabbit's serum. The result of this
test is shown in table 14.

TABLE 14

Determination of the precipitation titer of the pooled immune guinea-pig serum

IMMUNE GUINEA-PIG'S SERUM IN EACH TUBE	CUBIC CENTIMETERS OF RABBIT SERUM							
	0.1	0.05	0.03	0.01	0.005	0.0025	0.001	1.0005
cc. 0.1	Cloudy	Cloudy	+++	+++	++	+	±	—

With the mixed immune guinea-pigs' serum the "curative
experiment" of Weil (3) was carried out as follows: on April 18
three normal guinea-pigs received, by intraperitoneal injection,
0.3 cc. each of the serum of rabbit 440. (This rabbit had been
injected a number of times with egg white; the minimal sensitizing dose of its serum was 0.1 cc.) On April 19 each of the three
passively sensitized guinea-pigs was given 2 cc. of the pooled
immune guinea-pigs' serum by intravenous injection and two

days later the three animals were tested as to their hypersensitiveness to egg-albumin by intravenous injection. Two animals responded with very slight symptoms to injections of 1.0 cc. and 0.4 cc. respectively of the undiluted egg albumin solution;[6] the third animal died with the typical symptoms and anatomical changes of anaphylactic shock, after an injection of 0.6 cc. of the egg-albumin solution. The inflation of the lungs in this animal was not maximal.

This result confirms in a general way that obtained by Weil in the single animal employed in his experiment. It reveals, however, an irregularity in the "curative" action of the immune guinea-pigs' serum, which had escaped Weil and which is difficult to harmonize with Weil's view of the mechanism of antisensitization.

It is difficult, in other words, to explain why, notwithstanding the identical treatment of the three guinea-pigs, the "anti-antibodies" of the immune guinea-pigs' serum should have "neutralized" the antibodies of the sensitizing rabbit's serum in two animals but not in the third.

Having demonstrated the "antisensitizing" action of the mixed anti-rabbit guinea-pigs' serum with the technic of Weil's curative experiment, we were in position to examine the mechanism of that action with the test tube experiments. If, as Weil believed, the phenomenon of antisensitization is due to a specific interference by anti-antibodies, then it must be possible to precipitate the sensitizing antibodies from immune rabbit's serum by mixing the latter with the anti-rabbit guinea-pigs' serum *in vitro;* the antibodies of the rabbit's serum should be found to enter into the resulting specific precipitate as indicated by their absence in the supernatant fluid obtained by centrifugation.

On April 21, 12 cc. of the pooled anti-rabbit guinea-pigs' serum were mixed with 1.8 cc. of the anti-egg albumin serum of rabbit 440. This mixture was kept for one hour at 37°C. and then overnight in the ice-box. The precipitate which had

[6] The minimal lethal dose of this solution for guinea-pigs sensitized with three doses of anti-egg albumin immune rabbit's serum had been found to be not more than 0.003 cc.

formed was removed by rapid prolonged centrifugation and the clear supernatant fluid was examined: (1) As to its content of rabbit's serum proteins; (2) as to its power passively to sensitize against egg albumin; (3) as to its content of anti-egg albumin precipitins.

The examination for the presence of rabbit's serum proteins was made on April 22 by mixing 0.4, 0.2, 0.1, 0.05 and 0.025 cc. of the supernatant fluid with 0.1 cc. of the pooled anti-rabbit guinea-pigs' serum. In none of these mixtures had any precipitation taken place after they had stood overnight. All of the rabbit's serum protein that was precipitable with the anti-rabbit guinea-pigs' serum had been removed.

In examining the supernatant fluid as to its power passively to sensitize against egg albumin, account had to be taken of the dilution of the rabbit's immune serum (approximately 1–8) that had occurred as a result of mixing it with the guinea-pigs' immune serum.

On April 22 a series of normal guinea-pigs were given intraperitoneal injections of 0.8 cc. or 1.6 cc. or 2.3 cc. of the supernatant fluid; that is, amounts of the mixture representing 1, 2 and 3 minimal sensitizing doses of the immune rabbit's serum. On the following day the animals were tested with the intravenous injection of not less than 33 lethal doses of the egg albumin solution. The results of this test are presented in table 15.

TABLE 15

"Antisensitization" in vitro

GUINEA-PIG	AMOUNT OF THE SUPERNATANT FLUID USED FOR SENSITIZATION	AMOUNT OF EGG-ALBUMIN USED FOR TEST-INJECTION	RESULT
	cc.	cc.	
1	0.8	0.4	No symptoms
2	1.6	0.4	Slight symptoms
3	2.3	0.4	✠
4	2.3	0.4	✠
5	2.3	0.2	Slight symptoms
6	2.3	0.1	Slight symptoms

These results are susceptible of the interpretation that the effect of the anti-rabbit guinea-pigs' serum had been to increase threefold the sensitizing dose of the immune rabbit's serum and also to increase by more than 66 fold the killing dose of the egg albumin for the passively sensitized guinea-pigs. However, in view of the outcome of our "curative" experiment, it seems at least possible that in the test tube experiment we have encountered the same irregularity of action that we observed in the curative experiment; that is, it is possible that guinea-pigs number 5 and 6 would not have been killed with even 0.4 cc. of the egg albumin solution.

In any case, if Weil's hypothesis were correct it must have been possible to demonstrate, in the supernatant fluid, a change in the precipitating action against egg albumin corresponding with the change in the sensitizing action. In accordance with the results of our experiments in the partial neutralization of precipitin *in vitro* it could be expected that the change in the present instance would consist in an increase of at least 66 fold in the minimal precipitating amount of the egg albumin. However, the examination of the supernatant fluid in this respect showed no such change. Indeed, as the protocol of the comparative titration presented in table 16 shows, precipitation titer of the fluid corresponded exactly with that of the untreated rabbit's immune serum.

TABLE 16

Showing the comparative precipitation titer of the supernatant fluid and the original anti egg white rabbit's serum

	CUBIC CENTIMETERS OF EGG-ALBUMIN			
	0.002	0.0001	0.00002	0.00001
Supernatant fluid in each tube, 0.4 cc.......	+++	++	+(+)	+
Control (serum 140) in each tube, 0.05 cc...	+++	++	+(+)	+

The experiment just concluded demonstrates that the phenomenon of the anti-sensitization is not due to the interference of "anti-antibodies;" the power of the rabbit's precipitin of reacting with egg albumin was not perceptibly altered by contact

with the "anti-sensitizing" immune guinea-pigs' serum. Furthermore, the irregularity of the action of the anti-sensitizing guinea-pigs' serum suggests a non-specificity of that action which is in harmony with the observation of Weil that the active form of the anti-sensitization can be induced by the previous injection of heterologous sera.

III. EXPERIMENTS WITH SPECIFIC PRECIPITATES

In one of his studies in anaphylaxis (4) Weil attempted to demonstrate directly the identity of precipitin and sensitizing antibody ("sensitizin"). Weil's procedure was to inject washed specific precipitates into guinea-pigs and to test the injected animals, after a proper interval of time, as to the development of passive sensitization.

In such experiments Weil was usually successful, though in some instances the precipitate failed to sensitize the normal animals.

Our experiences with the partial neutralization of precipitin *in vitro* and *in vivo* made it seem unlikely that the combination of protein and precipitin would be more dissociable in the animal's body after the test-tube reaction than after the reaction that occurs *in vitro*.

We have, therefore, employed Weil's technic in an effort to duplicate his results. According to the procedure in his first experiment a constant amount of the precipitating serum—1.5 cc. —was mixed with different quantities of the antigen, crystalline egg albumin, and after an incubation of one hour at 37°C. and forty-eight hours in the ice-box, the mixtures were centrifugated and the precipitate was twice washed with 2 cc. of sterile saline solution. The whole of the washed precipitate from each mixture was injected intraperitoneally into a normal guinea-pig. Three days later each of the injected guinea-pigs was given a test injection of the egg albumin intravenously. None of the animals exhibited any symptoms of anaphylactic shock. Further details of this experiment are presented in table 17.

TABLE 17

Attempted passive sensitization with washed precipitates

SERUM 322 + 388 (MINIMAL SENSITIZING DOSE 0.1 CC.)	EGG-ALBUMIN	PRECIPITATE	WASHED PRECIPITATE INJECTED INTO GUINEA-PIG ON THIRD DAY	EGG-ALBUMIN INTRAVENOUSLY ON SIXTH DAY	RESULT
cc.	cc.			cc.	
1.5	0.001	+	1	1.0	No symptoms
1.5	0.005	+++	2	1.0	No symptoms
1.5	0.02	++++	3	0.6	No symptoms

This experiment was repeated with the sera of two rabbits that had been immunized with egg white. The results of these experiments are presented in table 18.

TABLE 18

Attempted passive sensitization with washed specific precipitates

Experiment II. $\begin{cases} \text{Serum 440} \\ \text{Serum 419} \end{cases}$ Immune serum v. egg white

	SERUM 440 MINIMAL (SENSITIZING DOSE) 0.1 CC.	EGG-ALBUMIN	PRECIPITATE	WASHED PRECIPITATE INJECTED INTRAPERITONEALLY INTO GUINEA-PIG ON THIRD DAY	EGG-ALBUMIN INTRAVENOUSLY ON SIXTH DAY	RESULT
	cc.	cc.			cc.	
	1.5	0.001	+	1	0.5	No symptoms
Series a	1.5	0.005	+++++	2	0.5	No symptoms
	1.5	0.02	++++	3	0.5	Moderate symptoms
	SERUM 440					
Series b	1.5	0.02	++++	4	0.5	No symptoms
	1.5	0.04	++++	5	0.5	No symptoms
	SERUM 419 MINIMAL (SENSITIZING DOSE) 0.1 CC.					
	1.5	0.001	+	6	0.5	No symptoms
Series c	1.5	0.005	++++	7	0.5	No symptoms
	1.5	0.02	++++	8	0.5	No symptoms

It is seen that a moderate sensitization was attained in only one animal and that when that test was repeated with the pre-

cipitate from an identical mixture (series B) not the least sensitization resulted. Thus, it appears that although our one animal may be looked upon as confirming Weil's observation of the passively sensitizing property of specific precipitates, the exhibition of that property is the exception rather than the rule.

The negative results appear the more striking when it is recalled that the precipitates were obtained from ten sensitizing amounts of the immune serum.

In the study of antibodies of various kinds, numerous efforts have been made to separate these from the serum proteins of the immune serum containing those bodies. Such studies have had either a practical end in view or they have sought information as to the chemical nature of the antibodies.

For technical reasons these efforts have been made with antibodies against formed antigens such as bacteria and blood corpuscles and the procedure has consisted in the absorption of the antibodies out of the immune serum with the antigenic elements followed by an extraction of the antigen-antibody combination with various substances at various temperatures. In the extraction fluid nearly all of the experimenters have demonstrated only the antibodies. Weil, alone, reported that he found antigen but no precipitating antibody in his "extracts." Weil mixed horse serum with the anti-horse serum of an immune rabbit and, following Chickering, he extracted the resulting precipitate with 1 per cent sodium carbonate. The extract, when mixed with 0.01 cc. of horse serum, remained clear, but when mixed with 0.5 cc. of the anti-horse serum, it formed a precipitate. This unique result was complicated by the successful *passive* sensitization of normal guinea-pigs, which Weil produced with the same extracts in which he could find no precipitin.

Weil explained this paradoxical outcome with the assumption of a haptophore or sensitizing group and an ergophore or precipitating group in the precipitin molecule.

We have investigated this phenomenon with an anti-egg white rabbit's serum. We mixed the serum with crystalline egg-albumin and dissolved the resulting precipitate, obtained by centrifugation and decantation of the supernatant fluid, in 2 cc. of

1 per cent sodium carbonate. In confirmation of Weil's obser-
vation it was found that the addition of 0.5 cc. of rabbit's immune
serum did produce a precipitate when mixed with a certain
amount of the carbonate solution of the precipitate. However,
this effect was shown not to be specific because an equal degree
of precipitation could be produced in the carbonate solution of
the precipitate with 0.5 cc. of a normal serum or by simply neu-
tralizing the extract. The phenomenon thus represented merely
a reprecipitation of a dissolved precipitate and not the specific
precipitation of free antigen, as Weil thought.

SUMMARY

1. Experiments are presented which demonstrate that pre-
cipitin remains unaltered quantitatively and qualitatively in the
guinea-pig for several days and that it reacts in the animal body
with its antigen in exactly the same manner as it does in the
test tube. The experiments furnish further evidence that pre-
cipitin and "sensitizin" are identical.

2. The phenomenon of "anti-sensitization" (Weil) is not due
to the action of anti-antibodies. It is a non-specific effect the
nature of which is obscure.

3. Passive sensitization with washed specific precipitates has
generally failed in our hands.

4. The sodium carbonate extract or solution of specific pre-
cipitates do not contain free antigen but represents the whole
precipitate in solution.

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THE PERFUSION EXPERIMENT IN THE STUDY OF ANAPHYLAXIS[1]

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The question as to the site of the specific protein-antiprotein reaction that is the original cause of the symptoms of anaphylaxis may be considered settled beyond reasonable doubt. The final acceptance of the "cellular theory" of anaphylaxis has been forced by unequivocal evidence in the case of the guinea-pig as well as of the dog. A detailed analysis of this evidence is in press elsewhere (1).

One of the arguments supporting the cellular theory was gained with the use of the process of perfusion, the purpose of which was to show that a once established hypersensitiveness persists after the blood contained in the vessels has been displaced with normal blood or saline solution.

To this end the perfusions have been conducted upon the living animal, either by means of the operation of direct transfusion, as in the pioneer experiments of Manwaring (2) and in the later ones of Pearce and Eisenbrey (3), or as in the writer's (4) experiments, by the infusion of defibrinated normal blood; the same object was sought by Dale (5) and by Manwaring and Kusama (6) with the perfusion of the isolated organs of sensitized animals.

The result of all of these experiments has been the same; the characteristic reaction of anaphylaxis has always been obtainable after the perfusion, however long the process has been continued. Nevertheless, these results have not been accepted by all as

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evidence of the cellular site of the anaphylaxis reaction on account of the lack of proof that every trace of blood is removable from the vessels with the procedure of perfusion. This doubt has been verified, in the case of the perfusion of isolated organs, by the recent experiments of Larson and Bell (7).

These investigators have found that if the perfusion of organs is interrupted, after the last traces of blood have disappeared in the fluid issuing from the organs, corpuscles and albumin reappear in the emerging fluid when the perfusion is resumed. The explanation of this phenomenon was drawn by Larson and Bell from further experiments, in which organs were perfused with fluid containing India ink. After thorough perfusion with such fluid, only limited portions of the organs were found to be blackened by the deposit of the ink particles in the phagocytic, fixed cells of those portions. The authors' explanation is that the perfusion fluid passed through only the blackened areas, which soon become free of blood. If "the perfusion is discontinued blood from the adjacent areas of (organ) tissue diffuses into the main path of the fluid. Hence, at each renewal of the perfusion after a pause, blood and albumin reappear."

The authors conclude that the "technic (of perfusion) does not therefore remove circulating antibodies completely, as has been assumed, and this type of experiment does not establish the presence of cellular antibodies."

These conclusions seem clearly to apply to the perfusion experiment as carried out with isolated organs, especially when the conditions of the experiment demand the *complete* removal of the blood by the perfusion. However, no evidence is presented by Larson and Bell to show the applicability of their conclusions to the perfusion experiment as conducted with the living animal.

In the writer's experiments with sensitized guinea-pigs it was not necessary for his purpose to displace all of the blood in the animal's body, but only so much as to leave *less than a single minimal sensitizing quantity* and according to his calculations this requirement was amply fulfilled. In the light of the experiments of Larson and Bell, it seems necessary, now, to see whether,

in the perfusion experiment with the living animal, also, the perfusion fluid reaches only limited areas of the organs.

This investigation was conducted with the simple procedure of injecting India ink, suspended in saline solution, into the circulation of a guinea-pig.   The animal was killed with chloroform and ether within two minutes after the injection and the organs were examined as to the distribution of the ink particles. The volume injected was 2 cc.   The injection was made into the external jugular vein.

FIG. 1.   Two Guinea-Pigs' Livers with their Respective Cross-Sections

The lighter one is from a normal animal; the darker one is from an animal that has received an injection of India ink.

A gross comparison was made of the organs of this animal with those of a guinea-pig that had not been injected.   The lungs of the injected animal were found to be of a slightly but uniformly greyer tint than the normal lung.   No distinct difference in the color of the other organs could be detected excepting the liver.   This organ, as can be seen in the photograph (fig. 1), was deeply blackened and uniformly so excepting a very narrow area near the anterior border on the inferior surface.

Section of the liver showed, also, a uniform distribution of the ink particles throughout the interior of the organ.

The experiment demonstrates an immediate, complete mixing of the injected fluid with the circulating blood and it shows that if any stagnant areas exist in the lung or liver these are not extensive enough to interfere with the purpose of the perfusion.

Thus, the perfusion experiments with live sensitized animals remain as uncontroverted evidence of the cellular site of the anaphylaxis reaction.

It may be pointed out that some of the experiments of Manwaring and Kusama, although they were carried out with an isolated organ, also contribute evidence of a cellular site of reaction, inasmuch as they show a distinctly increased anaphylactic reactivity after the flushing of the vessels in the case of the "immunized" guinea-pig. This experiment demonstrates the protective action of the circulating antibodies, which points directly to a cellular reaction site. In this experiment, also, a complete removal of the blood contained in the vessels of the organ was not necessary to the purpose.

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THE REACTION OF THE RAT TO DIPHTHERIA TOXIN

WITH OBSERVATIONS ON THE TECHNIC OF THE ROEMER METHOD OF TESTING DIPHTHERIA TOXIN AND ANTITOXIN

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It has been the practice of one of us to demonstrate to students the natural immunity of the rat to diphtheria toxin. This was done by injecting intraperitoneally 1 cc. of diphtheria toxin possessing a minimal lethal dose of 0.001 cc. Two rats were injected. One of these was kept under observation and always survived; the other was killed by bleeding on the morning following the injection and the whole quantity of the serum obtained was injected into a guinea-pig to demonstrate in the serum the presence of free toxin. The guinea-pig regularly died. This experiment showed that the immunity of the rat is not due to antitoxin in the animal's blood.

It was thought at first that since the rat can survive the injection of 1000 times the minimal lethal dose of toxin for the guinea-pig it must be absolutely immune to the toxin. Under this impression experiments were carried out to see whether the rat is capable of antitoxin production, as antitoxin production has not been demonstrated in animals that are absolutely immune to the respective toxin.

In examining the serum of the rats for the presence of antitoxin we have employed the method of Roemer which is adapted to the detection of very small quantities of toxin or antitoxin. In the course of our experience with this method some technical difficulties were encountered.

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FIGURE 1

The first difficulty had been met in preliminary experiments in the performance of the intracutaneous injection. In these experiments we had shaved the entire abdomen with a sharp razor—avoiding trauma—and had introduced the needle (27 gauge) into the somewhat tightly drawn skin in the manner employed for the Schick injection. With this manner of injection we were quite unable to control the depth of the injection. Before we began the present series of experiments we had learned from Dr. Abraham Zingher a method of injection, which, when properly applied, insures a truly superficial site of the injection. This method, which Dr. Zingher has not published, is illustrated in figure 1. The shaved skin is taken up in a loose fold over the forefinger and the needle is inserted superficially near the free border of the fold. The injection of 0.1 cc. of fluid in this situation always results in the formation of a tense vesicular swelling with a sharply defined base.

The second difficulty lay in the fact that occasionally in our experience the injection of identical material in different animals or even in different sites in the same animal produced widely different effect; for example, necrosis in one place, no necrosis in another.

In table 1 are shown the results of the preliminary test of the toxicity of the toxin with the use of the Roemer method.

It is seen that although in most of the animals necrosis was produced by both of the toxin dilutions, in guinea pig-285 this effect was lacking in one site even with the 1:600 dilution.

With this possible source of error in mind we conducted the earlier experiments with the use of duplicate injections of all mixtures into two animals. In accordance with the standard adopted for the usual subcutaneous method of injection, guinea-pigs had been selected weighing from 250 grams to about 300 grams, which is about the range of weight mostly observed by Roemer (1) in his original experiments. On account of the difficulty of maintaining a supply of animals of the selected range of weight some tests were carried out to see whether heavier animals could be used. In the first series of these tests the injections shown in table 1 were duplicated in six guinea-pigs

weighing between 430 and 630 grams. In this series there were no irregular results. Every injection produced necrosis. As it seemed possible that this somewhat surprising result was due to a greater susceptibility to the toxin on the part of the skin of the heavier animals, a further quantitative comparison was made, the results of which are presented in table 2.

It is seen that whereas in the heavier animals necrosis was regularly produced by 1/24,000 cc. of the toxin, in the smaller

TABLE 1

Preliminary test of the toxicity of diphtheria toxin with the use of Roemer's method. Result on the fourth day after intracutaneous injection of 0.1 cc.

GUINEA-PIG 289; WEIGHT 290 GRAMS	GUINEA-PIG 285; WEIGHT 260 GRAMS
L.P. 1 : 900 Necrosis 6 mm.	L.P. 1 : 900 Necrosis 7 mm.
R.P. 1 : 900 Necrosis 3 mm.	R.P. 1 : 900 0
L.A. 1 : 600 Necrosis 10 mm.	L.A. 1 : 600 0
R.A. 1 : 600 Necrosis 10 mm.	R.M. 1 : 600 Necrosis 10 mm.

GUINEA-PIG 286; WEIGHT 260 GRAMS	GUINEA-PIG 1849; WEIGHT 250 GRAMS
L.P. 1 : 90 Necrosis 3 mm.	L.P. 1 : 900 Necrosis 2 mm.
R.P. 1 : 90 Necrosis 3 mm.	R.P. 1 : 900 Necrosis 3 mm.
L.A. 1 : 600 Necrosis 7 mm.	L.A. 1 : 600 Necrosis 7 mm.
R.A. 1 : 600 Necrosis 7 mm.	R.A. 1 : 600 Necrosis 8 mm.

GUINEA-PIG 290; WEIGHT 250 GRAMS	GUINEA-PIG 479; WEIGHT 310 GRAMS
L.P. 1 : 900 Necrosis 7 mm.	L.P. 1 : 900 Necrosis 10 x 3 mm.
R.P. 1 : 900 Necrosis 7 mm.	R.P. 1 : 900 Induration
L.A. 1 : 600 Necrosis 10 mm.	L.A. 1 : 600 Necrosis 7 mm.
R.A. 1 : 600 Necrosis 10 mm.	R.A. 1 : 600 Necrosis 10 mm.

animals, corresponding with the earlier experiences, irregularity of effect was obtained with as much as 1/9000 cc.

It is evident in these experiments that the skin of the older guinea-pigs is more sensitive to the action of diphtheria toxin than the skin of the younger animals. For this reason the heavier animals must be preferred as test animals for the intradermal method. In numerous subsequent experiences this conclusion has been confirmed.

In examining the serum of the rats for the presence of anti-toxin the following procedure has been employed:

Serum was obtained by centrifugating the defibrinated blood and it was heated for thirty minutes at about 54°C. The toxin used throughout this study was furnished by Dr. Edwin J. Banzhaf of the Board of Health of New York City. The minimal lethal dose of this toxin was 1/400 cc. and the L_+ dose was 0.18 cc.

The toxin was used in a constant quantity in all of the mixtures; namely, 0.2 cc. of a 1 : 120 dilution. This quantity was somewhat less than 1/100 of the L_+ dose. Hence, failure of any certain quantity of serum to neutralize the toxin demonstrated that that quantity of serum contained not more than 1/100 of an antitoxin unit.

TABLE 2

Influence of the age of the guinea-pig on the result of Roemer's test for diptheria toxin
Result on fourth day after intracutaneous injection of 0.1 cc.

TOXIN DILUTION	GUINEA-PIG 213, WEIGHT 630 GRAMS	GUINEA-PIG 259, WEIGHT 290 GRAMS	GUINEA-PIG 280, WEIGHT 460 GRAMS	GUINEA-PIG 265, WEIGHT 240 GRAMS
1 : 2400	Necrosis	Scaling	Necrosis	Induration
1 : 1800	Necrosis	Scaling	Necrosis	Slight scaling
1 : 1500	Necrosis	Induration	Necrosis	Necrosis
1 : 900	Necrosis	Necrosis ?	Necrosis	Necrosis
TOXIN DILUTION	GUINEA-PIG 289, WEIGHT 580 GRAMS	GUINEA-PIG 286, WEIGHT 220 GRAMS	GUINEA-PIG 281, WEIGHT 550 GRAMS	GUINEA-PIG 283, WEIGHT 260 GRAMS
1 : 2400	Necrosis	Slight scaling	Necrosis	Scaling
1 : 1800	Necrosis	0	Necrosis	Slight necrosis
1 : 1500	Necrosis	Some scaling	Necrosis	Slight necrosis
1 : 900	Necrosis	Necrosis	Necrosis	0

The volume of the mixtures was always 1 cc. The quantity of mixture injected was always 0.1 cc. The animals were generally under daily observation and the final record of the results was made on the fourth day.

A number of preliminary tests had shown that in 0.8 cc. of normal rat's serum there is not enough antitoxin to prevent the necrotizing action of 0.2 cc. of a 1:120 dilution of the toxin. Following is a protocol of the experiments designed to determine whether the injection of diphtheria toxin into the rat results in antitoxin production.

August 11.   Eight white rats receive 0.5 cc. of diphtheria toxin each by subcutaneous injection.

August 18.   The eight rats injected on August 11 receive exactly similar injections.

August 25.   Six of the injected rats receive a third injection exactly similar to the first.   The other two rats are etherized and bled to death from the heart.   The defibrinated blood is pooled and the serum, obtained by centrifugation, is heated for 30 minutes at 54°C.   0.8 cc. of this serum did not interfere with the necrotizing action of 0.2 cc. of toxin diluted 1:120.

September 1.   One of the injected rats is bled and the heated serum is examined as usual.   No antitoxic action is demonstrable.   The remaining rats receive the fourth injection of 0.5 cc. of toxin.

September 8.   One of the injected rats is bled and the heated serum is examined.   0.4 cc. of this serum are found to prevent completely the necrotizing action of 0.2 cc. of toxin (1 : 120).   Smaller quantities of the serum are not tested.   The remaining rats receive the fifth injection of 0.5 cc. of toxin.

September 15.   The serum of one of the injected rats is obtained and tested as usual.   One-tenth of a cubic centimeter of the serum is found to neutralize completely 0.2 cc. of toxin (1 : 120).   1/40 cc. exhibited no neutralizing effect.   The remaining rats receive the sixth injection of 0.5 cc. of toxin.

September 22.   The serum of one of the injected rats was obtained and heated as usual.   1/40 cc. of this serum prevented the necrotizing action of the usual quantity of toxin but allowed some sealing.   1/160 cc. of the serum exhibited no antitoxic power.

These experiments show that the white rat, although insensitive to the injection of 1000 times the lethal dose of diphtheria toxin for the guinea pig can produce antitoxin after four injections of 200 doses.   After the sixth injection the serum of the last rats examined contained at least 4/10 of an antitoxin unit per cubic centimeter.

The foregoing experiments seemed to indicate that an animal can produce antitoxin against a toxin to which it is not susceptible. It did not seem likely that an animal which was able to receive 1000 lethal doses of toxin without exhibiting symptoms would be intoxicated by a larger quantity of the toxin.

Nevertheless, the following tests were carried out as a precautionary measure:

October 18. 14 cc. of diphtheria toxin had been mixed with 0.2 cc. (100 units) of diphtheria antitoxin and the mixture had been kept in the ice-box. 4 cc. of this overneutralized toxin were injected subcutaneously into each of three normal white rats. Three other normal rats received 4 cc. of each of toxin with which no antitoxin had been mixed. All of the six animals were etherized, as usual, before the injections, but they did not recover as quickly as usual and all of them exhibited twitchings of the whole body for a considerable time. Eventually, however, all recovered and on the following day they seemed normal, with the exception of one of the animals that had received the unneutralized toxin. This animal died during the day. Autopsy showed slightly congested adrenals and some excessive fluid in the abdominal cavity.

The other two animals that had received the unneutralized toxin died on October 21 and 22 and both presented slightly but distinctly congested adrenal glands. All of the rats that received the neutralized toxin survived without symptoms.

The foregoing experiment demonstrates that the rat is not entirely immune to diphtheria toxin: it demonstrates a slight degree of susceptibility which corresponds with the slight power of antitoxin production in this animal.

THE MECHANISM OF THE NATURAL RESISTANCE OF THE RAT TO DIPHTHERIA TOXIN

Our knowledge regarding the natural cellular immunity to toxin is limited almost entirely to the assumption of Ehrlich that immune cells lack the appropriate "receptors" through which alone, according to him, toxin can become effectively attached to cells. However, in the one instance in which it has been possible to put this assumption to experimental test the theory has been shown to be inapplicable.

Suspended in a medium containing electrolytes, the red blood corpuscles of the ox and the sheep are entirely immune to the action of cobra hemotoxin. If these corpuscles are suspended in isotonic solutions of sugars (2) in the complete absence of elec-

trolytes they are found to be susceptible to cobra hemotoxin. The simple addition of electrolyte to the sugar medium restores the cellular immunity.

It has been found that in a medium containing electrolytes the resistant corpuscles are unable to absorb the cobra hemotoxin, whereas in a medium free from electrolytes absorption of a considerable quantity of hemotoxin can be demonstrated.

These facts show that the immunity of the resistant corpuscles is due to a physical condition of the corpuscular substance, which prevents the entrance of the toxin. This physical condition is altered by the mere exclusion of electrolytes, by which means the corpuscles become permeable to the toxin.

The assumption suggested itself that the mechanism of the natural corpuscular resistance to cobra hemotoxin is operative in all instances of natural cellular immunity to toxin, including, therefore, that of the rat to diphtheria toxin. The truth of this assumption could not be tested in the present instance by the exclusion of electrolytes in the animal experiment—it was impossible to find out whether in the absence of electrolytes the rat's tissues would become more susceptible to the action of diphtheria toxin.

However, evidence regarding the mechanism of the rat's immunity could be sought by another means. It was evident that if a fixed quantity of toxin be injected into an animal the concentration of the toxin in the body fluids will be affected according to whether the toxin permeates the cells or not. This principle has been used by Hedin (3) and later by Kosakai (4) to determine the permeability of blood corpuscles for various substances; it was applied in the present study as follows:

A certain quantity of diphtheria toxin per 100 grams of animal weight was injected intraperitoneally into a rat and into a guinea-pig and after six to seven hours the animals were etherized and the toxin content of their serum was determined by subcutaneous injection of the sera into guinea-pigs.

If the rat's cells are only slightly permeable to the toxin so that most of the toxin remains in the body fluids the serum of the

rat obtained in this experiment should be more toxic than that of the guinea-pig, whose cells are highly susceptible and therefore easily permeable to the toxin. The results of the experiments presented in the following protocols amply meet these hypothetical conditions.

Protocol 1

Rat 1, weighing 250 grams and guinea-pig 1, weighing 220 grams, received respectively 2.5 cc. and 2.2 cc. of diphtheria toxin by intraperitoneal injection. Six hours later both animals were etherized and bled from the heart. The sera, obtained by centrifugation of the defibrinated blood were injected subcutaneously into normal guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	grams		*cc.*	
265	250	Serum rat 1 (1 : 20)	1.0	Died in 4 days
256	250	(Same)	0.5	Local edema: S.
273	210	Serum guinea-pig 1 (1 : 20)	2.0	Died in 3 days
274	240	(Same)	1.0	Local edema: S.

S = survived.

Protocol 2

Rat 2, weighing 310 grams and guinea-pig 2, weighing 275 grams, receive respectively 1 cc. and 0.9 cc. of diphtheria toxin by intraperitoneal injection. Six hours later the animals were bled and the serum obtained was injected into guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	grams		*cc.*	
251	250	Serum rat 2	0.5	Died in 2½ days
249	250	(Same)	0.3	Died in 2½ days
215	290	Serum guinea-pig 2	2.0	Died in 2 days

Guinea-pig 2a weighing 280 grams was treated like guinea-pig 2 and 1 cc. of the serum, obtained as usual after seven and one-half hours, was injected into normal guinea-pig 252, weighing 290 grams. This animal died on the fourth day.

Protocol 3

Rat 3 (320 grams) receives 0.64 cc. and guinea-pigs 3, 4 and 5, weighing 270, 270 and 250 grams, receive respectively 0.54 cc., 0.54 cc. and 0.50 cc. of diphtheria toxin by intraperitoneal injection. Six and a half hours later all of the animals were bled and the sera obtained from the three guinea-pigs were pooled. The rat's serum and the pooled guinea-pigs' serum were injected into normal guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	grams		*cc.*	
463	250	Serum of rat 3	0.8	Died in 3½ days
462	250	Serum of rat 3	0.5	Died in 4½ days
423	260	Serum of rat 3	0.5	Died in 3 days
427	About 250	Serum of rat 3	0.4	Died in 4 days
428	240	Serum of rat 3	0.35	Died in 14 days
429	260	Serum of rat 3	0.3	Lived
464	260	Pooled serum guinea-pigs 3, 4, 5	3.0	Died in 2½ days
426	260	Pooled serum guinea-pigs 3, 4, 5	2.0	Died in 4 days

Protocol 4

Rat 4 (170 grams) received 1.7 cc. of diphtheria toxin intraperitoneally and rats 5, 6 and 7, weighing 160 grams, 200 grams and 200 grams, received respectively 0.32 cc., 0.4 cc. and 0.4 cc. of toxin intraperitoneally. Between six and six and one-half hours later all of the animals were bled and the sera were obtained as usual. Serum 4 was kept separate; sera 5, 6 and 8 were pooled. These two sera were injected, subcutaneously into normal guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	grams		*cc.*	
456	260	Serum rat 4 (1 : 10)	0.6	Survived
450	250	(Same)	0.7	Survived
452	250	(Same)	0.8	Died in 4½ days
322	260	(Same)	0.8	Died in 5½ days
324	255	(Same)	0.9	Died in 4 days
323	260	(Same)	1.0	Died in 2½ days
327	275	Serum rats 5, 6 and 7, undiluted	0.7	Died in 4½ days
316	250	(Same)	0.8	Died in 2 days
320	250	(Same)	1.0	Died in 2 days
458	250	(Same)	1.0	Died in 3 days

Protocol 5

Guinea-pig 317 (250 grams) received intraperitoneally 2.5 cc. of diphtheria toxin. Guinea-pigs 318, 319 and 321, each weighing 250 grams, received 0.5 cc. of diphtheria toxin each by intraperitoneal injection. Nearly seven hours later these animals were bled and the sera were obtained as usual. Serum 317 was kept separate; sera 318, 319 and 321 were pooled. The two sera were injected subcutaneously into normal guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	grams		cc.	
452a	250	Serum guinea-pig 317	0.2	Died in 11 days
326	250	(Same)	0.3	Died in 4 days
37a	240	Serum guinea-pigs 318, 319 and 321	1.6	Died in 7 days
325	250	(Same)	1.8	Died in 3 days
321a	240	(Same)	2.0	Died in 3 days

In the preliminary experiment presented in protocol 1 it was found that six hours after the injection of 1 cc. of toxin per 100 grams the minimal lethal dose of the rat's serum was about 0.05 cc.; that of the guinea-pig's serum was about 1 cc. The results of more exact determinations of the toxicity of the sera under the conditions of this preliminary experiment are given in protocols 5 and 6. The lethal dose of the corresponding rat serum 4 was found to be 0.09 cc.; that of the corresponding guinea-pig's serum 317 was 0.3 cc. The difference in the toxicity of the two sera is decidedly greater in this experiment than it was in the first one.

Still greater differences in toxicity were observed when the quantity of toxin injected per 100 grams was less than 1 cc. For example, the lethal dose of the serum of rat 3 (which had received 0.2 cc. per 100 grams) was 0.4 cc., whereas that of the corresponding pooled guinea-pigs' sera 3, 4 and 5 was five times as much—2 cc.

The foregoing experiments seem to indicate that the cells of the rat are usually much less permeable to diphtheria toxin than are those of the guinea-pig. However, it is also conceivable that

the toxin does not actually permeate the cells of the susceptible guinea-pig, but merely attaches itself to a vulnerable element or "receptor" on the surface of the cells. The failure of this attachment to take place in the case of the resistant animal could account for the differences recorded in our experiments.

There is, thus, nothing in our results which can be considered as actually invalidating the side chain theory. All that can be asserted regarding the mechanism of the rat's immunity to the toxin is that the cells of this animal seem to possess the property of preventing the toxin from permeating them or of attaching itself to them.

SUMMARY

1. Zingher's method of injecting intracutaneously in guinea-pigs is described.

2. It is shown that constant results can be obtained with the Roemer technic only if the larger guinea-pigs are used (over 400 grams).

3. The rat is not absolutely immune to diphtheria toxin. Although it usually survives the injection of 1000 minimal lethal doses (for the guinea-pig) it regularly succumbs to 4000 such units.

4. The rat is capable of the production of antitoxin upon the repeated injection of diphtheria toxin.

5. The resistance of the rat to diphtheria toxin is not due to the presence of normal antitoxin, but to the property of the cells of preventing the toxin from entering them or of attaching itself to them.

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STUDIES IN OSMOTIC PRESSURE

I. THE MECHANISM OF BORIC ACID HEMOLYSIS

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Of the long list of the studies of the phenomena of the hemolysis almost all have been concerned with the investigation of the properties of the various hemolytic agents, very few having pursued the question of the actual force that brings about the destruction of the corpuscles. Indeed, this ultimate force is known in but one of the many forms of the process of hemolysis that resulting from hypotonicity of the medium of suspension; that is, osmotic pressure.

In the course of some experiments upon the preservation of blood corpuscles with boric acid A. F. Coca made the following observations:[1] boric acid in dry form or in solution may be mixed with blood in a proportion of $1\frac{1}{2}$ per cent, or less, of the dry substance without causing hemolysis. If blood that has been in contact with boric acid for a time is suddenly mixed with one or more volumes of 0.9 per cent NaCL solution, immediate complete hemolysis occurs. If the blood that has been treated with boric acid is mixed with more concentrated solutions of sodium chloride no hemolysis takes place.

As the easily controlled conditions leading to this phenomenon appeared to be favorable for the discovery of the ultimate mechanism of the hemolytic process, the present further study of it was undertaken.

[1] Unpublished.

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TECHNIC

The corpuscle suspension used throughout this investigation was prepared from oxalated sheep's blood by washing the latter three times in physiological (0.9 per cent) NaCL solution and diluting the corpuscular sediment with the same solution up to the original volume of the blood taken.

The concentration of the solutions of all of the substances employed was determined by titration so far as methods of titration for this purpose were available.

The glass tubes used for the hemolysis tests were of ordinary American glass and they measured 7 cm. in length by about 1 cm. in diameter.

The experiments were carried out at room temperature (20° to 24°C). The influence of ordinary changes of temperature upon the results was quite negligible.

The direct effect of boric acid upon the corpuscles was first examined and it was found that, in the stronger concentrations, this substance is capable, after a period of time, the length of which is influenced directly by its concentration, of producing complete or nearly complete laking. The protocol of an experiment showing the direct hemolytic action of boric acid is presented in table 1.

That this direct hemolytic effect is not due to the acid quality of the substance; that is, to the action of hydrogen ions, seems deducible from the fact that boric acid is not dissociated in solution and also from the absence of methemoglobin formation, which is always observed after hemolysis with mineral acids and dissociable organic acids.

The preceding experiment does not exclude the possibility that, although the direct boric acid hemolysis depends on the *concentration* of the reagent in the original mixture, the phenomenon is influenced, also, by the quantitative relation between the corpuscles and the boric acid. This question was investigated by preparing two mixtures of equal volume containing an identical amount of boric acid but different amounts of corpuscles. The boric acid concentration used was such that

the usual amount of the corpuscle suspension (0.05 cc., tube
A) was not completely hemolysed.

If the degree of hemolysis were affected by the quantitative
relation between the corpuscles and the reagent, then less
hemolysis would be expected in the mixtures containing the
larger amounts of corpuscles (that is 0.1 cc., tube B). How-
ever, the degree of hemolysis was identical in the two mixtures.
On the other hand, the hemolytic effect was distinctly less in
a mixture in which the quantitative relationship between cor-

TABLE 1

Showing the direct hemolytic action of boric acid

	NUMBER OF TEST TUBE				
	I	II	III	IV	V
Boric acid solution (3.5 per cent), cc..........	0.3	0.5	0.95	1.95	2.95
Physiological salt solution (cc.)..............	0.65	0.45			
Blood suspension (cc.).......................	0.05	0.05	0.05	0.05	0.05
	Degree of resulting hemolysis				
8 minutes.................................	0	0	0	0	c
12 minutes.................................	0	0	0	c	c
2.5 hours....................................	0	tr	alc	c	c
20 hours....................................	0	tr	alc	c	c

In all of the tables the following abbreviations are used to indicate the de-
gree of the resulting hemolysis: c = complete, alc = almost complete, v.st. =
very strong, st = strong, w = weak, v.w = very weak, tr = trace, 0 = no hemoly-
sis.

puscles and boric acid was the same as that of mixture B but
in which the concentration of the reagent was only half as great
as in the latter mixture.

From the results of the first experiment it is seen, furthermore,
that in the lesser concentrations (tubes 1 and 2) boric acid
exerts little or no hemolytic action. These concentrations
were, therefore, suitable for the demonstration of the phenome-
non of hemolysis observed by Coca. For this purpose a con-
stant amount of corpuscular suspension was mixed with boric
acid in the strongest concentration that was not directly hemo-

lytic and also in three lesser concentrations. After five minutes an equal volume (1 cc.) of 0.9 per cent NaCI solution was added to each mixture and the tubes were at once vigorously shaken.

The results of this experiment are presented in table 2.

It is seen that complete hemolysis occurred only in the mixture containing the stronger concentration of the boric acid; that is, *where the relative change in the concentration of that substance caused by the addition of the salt solution was greatest.* Furthermore, a significant characteristic of the boric acid hemolysis[2] is seen in the results of this experiment, in the fact that *the full hemolytic action elicited by the procedure is exerted*

TABLE 2

	NUMBER OF TEST TUBE			
	I	II	III	IV
Boric acid solution (3.5 per cent) (cc.)..............	0.5	0.4	0.3	0.2
Physiological salt solution (cc.).....................	0.45	0.55	0.65	0.75
Blood suspension (cc.).............................	0.05	0.05	0.05	0.05

After five minutes, 1 cc. of physiological salt solution is added into each tube

Result { At once..	c	w	tr	0
After ten hours...............................	c	w	tr	0

within a few seconds, not the least further hemolysis occurring in any of the tubes during the succeeding ten hours.

Since the cause of the hemolysis appeared to depend upon the sudden change of the concentration of the boric acid in the medium of suspension of the corpuscles it seemed possible that the addition of more than an equal volume of salt solution would cause hemolysis, also, in the mixtures containing lesser concentrations of boric acid and this was found to be true. When to the mixtures in tubes 2 and 3 in table 2 several volumes of 0.9 per cent NaCI solution were added complete hemolysis took place in both instances.

[2] In the further discussions in this paper the term ''boric acid hemolysis'' refers to the phenomenon of hemolysis observed by Coca, not to the direct hemolysis produced by the concentrated reagent.

In view of these latter results it could be anticipated that the addition to the mixture in tube 1 of less than an equal volume of salt solution could result in a correspondingly lesser degree of hemolysis. That this is true is shown in the protocol presented in table 3.

It is seen that when a half volume of salt solution was added to the mixture only weak hemolysis occurred, the addition of a three-tenths volume causing only a trace of hemolysis.

Similarly, it was anticipated that if the mixtures in the tubes 2 and 3 of table 2 were centrifuged and the supernatant fluid decanted, hemolysis could then be produced by shaking the sediment in a relatively small volume of NaCI solution—one

TABLE 3

	NUMBER OF TEST TUBE		
	I	II	III
Boric acid solution (3.5 per cent) (cc.).....................	0.5	0.5	0.5
Physiological salt solution (cc.)............................	0.45	0.45	0.45
Blood suspension (cc.)......................................	0.05	0.05	0.05

After five minutes was added to I, 1.0 cc. of physiological salt solution; to II, 0.5 cc. of physiological salt solution; to III, 0.3 cc. of physiological salt solution.

Result {	At once..	c	w	tr
	After ten hours.....................................	c	w	tr

which, without centrifugation, would fail to cause laking. The results of such an experiment, which are given in table 4, show this to be the case.

That the hemolysis produced by the addition of salt solution to blood that has been treated with boric acid is not due to a specific action of sodium chloride is demonstrated by the fact that hemolysis occurs as in all of these circumstances if, instead of the solution of sodium chloride isotonic solutions of other salts or of sugar are added to the blood corpuscle and boric acid mixtures. Indeed, the same phenomenon is observed upon the addition of sheep's serum to those mixtures.

The data in hand made it appear possible that the *concentration* of the boric acid rather than its absolute quantity in

3. The boric acid hemolysis occurs after a sudden lowering of the concentration of the reagent in the medium of suspension of the corpuscles.

The only force that is known to be developed under the foregoing conditions is that of osmotic pressure, which, of course, must be assumed to act upon a limiting corpuscular cell membrane and the further evidence, which will be presented, leaves no reasonable doubt that this force is, in fact, the ultimate cause of the phenomenon of hemolysis that we are studying.

The assumption that the boric acid hemolysis is an effect of osmotic pressure necessitates the further assumption that the reagent is able to permeate the corpuscular membrane. As is well known (1) the corpuscular membrane is permeable to many substances and impermeable to others. Since boric acid had not yet been studied in this respect, it was necessary to determine the question by experiment. For this purpose the method of Hedin was employed as follows: To 10 cc. of washed sheep's blood, made up to the original volume with physiological saline solution, were added 10 cc. of physiological saline solution containing 3.5 per cent of boric acid. After 10 minutes this mixture was centrifuged and the supernatant fluid was compared with a mixture of 10 cc. of physiological saline solution and 10 cc. of the boric acid-saline solution as to its osmotic concentration.

If the boric acid was capable of entering the corpuscles and was present there in the same concentration as in the medium of suspension, then the osmotic concentration in the fluid medium was, of necessity, the same as that of the mixture without the corpuscles. On the other hand, if the corpuscular membrane was impermeable or incompletely permeable to the boric acid, then the osmotic concentration in the medium of suspension must have been greater than it was in the mixture without the corpuscles. The osmotic concentration in the two fluids was determined with the cryoscopic method, with the following result:

1. Supernatant fluid of corpuscle mixture..............................1.120
2. Control mixture without corpuscles...............................1.122

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The distribution of the boric acid in the suspension of corpuscles is, thus, uniform throughout the corpuscles and the medium of suspension.

GRADUAL ADDITION OF PHYSIOLOGICAL SALINE SOLUTION TO "BORATED" CORPUSCLES

If the force that is operative in the boric acid hemolysis is purely that of osmotic pressure and if the corpuscles are not directly injured by the mere contact with the reagent, it should be possible, by the gradual addition of a volume of salt solution that, otherwise, is hemolytic, to bring about a slow diffusion of the intracorpuscular boric acid into the surrounding medium without the production of hemolysis. The difference between the intracorpuscular and the extracorpuscular concentration of the boric acid should, by this procedure, easily be kept below the point at which the assumed destructive osmotic pressure is developed. The protocol of the experiment that was conducted according to this plan is presented in table 4.

The result of the experiment is in harmony with the "osmotic pressure" theory of the boric acid hemolysis and it demonstrates that the hemolytic effect is not caused by a direct injury of the corpuscles by the reagent.

THE INHIBITION OF THE BORIC ACID HEMOLYSIS WITH CONCENTRATED SOLUTIONS OF ELECTROLYTES AND NON-ELECTROLYTES

The already cited observation of Coca, that the boric acid hemolysis can be inhibited if, in the second step of the procedure concentrated NaCl solution is substituted for the 0.9 per cent NaCI solution, is also compatible with the osmotic pressure theory. This phenomenon was subjected to a quantitative study; the protocol of the preliminary experiments is presented in table 5.

It is seen that corpuscles which have been treated with boric acid in the manner indicated can be mixed suddenly with 1 cc. of a 1.4 per cent or more concentrated solutions of sodium chlorid

without undergoing the least degree of hemolysis.   In concentrations of 1 per cent or less the corpuscles are completely laked.

In terms of the osmotic pressure theory it could be assumed that certain concentrations of sodium chlorid are able to offer a counter osmotic pressure upon the external aspect of the corpuscular membrane which neutralizes that developed by the boric acid within the cell.   It could be assumed, furthermore, that those concentrations of sodium chlorid need not prevent the diffusion of the boric acid out of the corpuscles and experimental examination of this question, the protocol of which is presented in table 6, shows that such is the case.

TABLE 5

Each tube contains: Boric acid solution (3.5 per cent), 0.3 cc.; physiological salt solution, 0.65 cc.; blood suspension 0.05 cc. After five minutes, all centrifuged, and 1.0 cc. of the solutions of sodium chloride added.

	NUMBER OF TEST TUBE							
	I	II	III	IV	V	VI	VII	VIII
Concentration of sodium chloride (per cent)......................	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6
Result { At once...................	c	c	v.st	w	tr	0	0	0
After two hours...........	c	c	v.st	w	tr	0	0	0

The insensitiveness of the corpuscles, after the second centrifugation, to sudden immersion in physiological saline solution indicates that the concentration of boric acid remaining in them was no longer great enough to develop a destructive osmotic pressure.   Moreover, the result of this experiment furnishes clear evidence that the boric acid hemolysis is not due to any organic change in the corpuscles caused by a chemical action of the reagent.

In order to avoid confusion resulting from discrepancies that may appear to exist, with respect to hemolytic effect, in the quantitative relations in the different protocols, it may be stated that the different specimens of sheep's corpuscles have been found to be differently susceptible to the hemolytic influence of boric acid that we are considering; that is, some speci-

mens of blood could be completely hemolysed after treatment
with concentrations of boric acid, which, with other speci-
mens of blood, induced only a partial hemolysis. This factor
has sometimes interfered with a direct comparison of the re-
sults in the different protocols, but it has, in no way, detracted
from the conclusions of the study, which have been based on
individual experiments.

If the absence of hemolysis upon the immersion of borated
corpuscles in a concentrated solution of sodium chlorid is due

TABLE 6

*Each tube contains: Boric acid solution (3.5 per cent), 0.3 cc.; physiological salt
solution, 0.65 cc.; blood suspension, 0.05 cc. After five minutes all tubes were
centrifuged and the supernatant fluid in each was decanted*

	NUMBER OF TEST TUBE			
	I	II	III	IV
Solution added...... {	Physiological salt solution 1.0 cc.	1.5 per cent NaCl 1.0 cc.	2.0 per cent NaCl 1.0 cc.	2.5 per cent NaCl 1.0 cc.
Result..........	c	0	0	0

After five minutes tubes II, III and IV again centrifuged and the sediment in
each was mixed suddenly

Physiological salt solution..				
Result..........		3 cc.	3 cc.	3 cc.
		0	0	0

to a counter osmotic pressure developed against the outer sur-
face of the corpuscular membrane, then a similar effect must
be producible with suitably concentrated solutions of other
substances capable of inducing osmotic pressure. Further-
more, if the theory under consideration is correct, it must be
possible to demonstrate that the minimal non-hemolytic con-
centrations of all such substances for corpuscles that have been
treated with a certain concentration of boric acid actually
exert the same osmotic pressure. In the succeeding experi-
ments these requirements are fully satisfied and the concor-

dant results permit the definite conclusion that *the force opera-*
tive in the boric acid hemolysis is, in fact, that of osmotic pressure.

In these experiments the treatment of the corpuscles was
always carried out in a volume of 1 cc. of the different concentra-
tions of the boric acid. The concentration of the boric acid is
usually indicated in the tables by the amount of a 3.5 per cent
solution of that reagent that was contained in the treating
mixture. For example, where the amount of boric acid is
given as 0.15, this means that in making that mixture 0.15 cc.
of 3.5 per cent of boric acid dissolved in physiological saline
solution were mixed with 0.8 cc. of physiological saline solution
and to this mixture were added 0.05 cc. of blood suspension.

In order to determine the minimal non-hemolytic concen-
tration of the different substances, five or six identical mix-
tures of blood and boric acid were prepared and after five minutes
the mixtures were centrifuged and the supernatant fluid was
completely removed with a capillary pipet. With the sedi-
ment of each tube was rapidly mixed (by shaking) one cubic
centimeter of the different concentrations of the substance
under examination. Within a few minutes these final mix-
tures were centrifiged and the degree of the resulting hemolysis
was noted according to the degree in which the supernatant
fluid was tinged with hemoglobin. The minimal non-hemolytic
concentration was taken as the lowest with which no tinging
of the supernatant fluid resulted. The relative osmotic pres-
sure of the various concentrations was determined with the
usual cryoscopic method, the results of these examinations
being recorded in the tables under the customary designation.

In a preliminary experiment the relation of the osmotic pres-
sure of the treating mixture to that of the minimal inhibiting
concentration of pure sodium chlorid and of boric acid dis-
solved in physiological sodium chlorid was studied and, as the
tabulated protocols (tables XIII and XIIIa) show, it was found
that the "minimal inhibiting pressure" was slightly, but con-
sistently lower than the "treating pressure." It is impossible
to interpret this difference without exact information as to the
balance of osmotic pressure between the normal corpuscular

contents and the "isotonic" saline solution in which the boric acid, used in the experiment, had been dissolved. A part if not all of the difference represents variation due to experimental error.

It is seen, furthermore, that for corpuscles that have been similarly treated with boric acid the minimal non-hemolytic

TABLE 7

Showing the method of determining the minimal concentrations of a substance (NaCl) which are capable of preventing the boric acid hemolysis

	NUMBER OF GROUP							
	I	II	III	IV	V	VI	VII	VIII
Amount of boric acid solution (3.5 per cent) used for treatment.............	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
Solution of NaCl, per cent	Degree of resulting hemolysis							
0.9	st	c						
1.0	w	st	alc					
1.1	0	w	st	alc				
1.2	0	0	w	st	c			
1.3	0	0	0	w	st	c		
1.4		0	0	0	w	st	c	
1.5					0	w	alc	
1.6						tr	st	alc
1.7						0	v.w	st
1.8						0	0	v.w
1.9							0	0
2.0								0

concentration of sodium chlorid and of boric acid are found, with the method employed, to be of practically identical "osmotic concentration."

In two further series of tests, the results of which are presented in tables 9 and 10, the "osmotic concentration" of the minimal non-hemolytic concentrations of four other substances was determined for differently treated corpuscles. As different

TABLE 8

Relation of the concentration of boric acid used in treatment to the minimal inhibiting concentrations of sodium chloride and boric acid

TREATING CONCENTRATION OF BORIC ACID			INHIBITING CONCENTRATION			
Amount of a 3.5 per cent solution in 1.0 cc. of original mixture	Per cent	Δ	NaCl		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ
cc.						
0.5	1.75	1.115	1.85	1.095	1.54	1.07
0.45	1.575	1.075	1.75	1.05	1.40	1.00
0.4	1.4	1.00	1.62	0.97	1.275	0.95
0.35	1.225	0.94	1.5	0.91	1.05	0.88
0.3	1.05	0.88	1.4	0.85	0.945	0.84
0.25	0.875	0.835	1.3	0.83	0.77	0.80
0.2	0.7	0.78	1.18	0.74	0.595	0.75
0.15	0.525	0.73	1.08	0.69	0.42	0.67

TABLE 9

Relative osmotic concentration of the minimal non-hemolytic concentrations of sodium chlorid, barium chlorid and cane sugar

AMOUNT OF 3.5 PER CENT BORIC ACID USED FOR TREATING THE CORPUSCLES	NON-HEMOLYTIC CONCENTRATION OF					
	NaCl		BaCl₂		Cane sugar	
	Per cent	Δ	Per cent	Δ	Per cent	Δ
cc.						
0.2	1.2	0.749	3.7	0.724	11.0	0.722
0.3	1.4	0.850	4.4	0.858	13.0	0.866
0.4	1.6	0.950	4.9	0.971	14.0	0.964
0.5	1.8	1.082	5.4	1.076	15.0	1.125

TABLE 10

Relative osmotic concentration of the minimal non-hemolytic concentrations of ammonium chlorid and glycerin

AMOUNT OF 3.5 PER CENT BORIC ACID USED FOR TREATING THE CORPUSCLES	NON-HEMOLYTIC CONCENTRATION OF			
	NH₄Cl		Glycerin	
	Per cent	Δ	Per cent	Δ
cc.				
0.2	1.2	0.801	2.9	0.794
0.4	1.5	1.010	3.8	1.041

specimens of corpuscles were used in the two series of tests, the results in the two tests are not concordant. Those of table 9 agree with those of table 8, both of these disagreeing with those of table 10. However, the results included within each table are concordant among themselves and they allow no reasonable doubt that *the prevention of the boric acid hemolysis with concentrated solution is due to a counter osmotic mechanism acting upon the outer surface of the corpuscles.*

SUMMARY

After blood corpuscles have been treated for a short time with certain concentrations of boric acid that are not directly injurious to those cells, the sudden immersion of the treated corpuscles in a physiological solution of sodium chloride causes their complete hemolysis.

This "boric acid hemolysis" does not occur if the addition of the physiological saline solution is made gradually or if the corpuscles are immersed, even suddenly, in more concentrated solutions of sodium chloride or of other non-hemolytic substances.

That the destructive force responsible for this form of hemolysis is that of "osmotic pressure" is shown by the fact that the minimal non-hemolytic concentrations of all of the substances examined were found to be of identical "osmotic concentration."

REFERENCE

(1) HEDIN: Grundzüge der physikalischen Chemie, Wiesbaden, 1915, p. 22.

STUDIES IN OSMOTIC PRESSURE

II. THE NATURE OF OSMOTIC PRESSURE

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The laws governing the development of osmotic pressure
and the effects produced by that agency upon animal and
vegetable cells have been exactly determined by experimental
study. The nature of the force of osmotic pressure, on the
other hand, has, of necessity, been only surmised, because of
the limitations hitherto surrounding the experimental study
of that force, and the ideas concerning this question are based
either on theoretical grounds or on the observations upon the
effects of osmotic pressure.

In the writings on the subject of osmotic pressure two con-
ceptions of the nature of that force are found.

The first conception is drawn from the well known demon-
stration by van't Hoff of the agreement between the laws
governing gas pressure and those governing the development
of osmotic pressure. According to this conception, osmotic
pressure, corresponding with the pressure of the gases, is ex-
erted by the molecules of the dissolved substance (solute), these
being thought of as continually "bombarding" the surface of
the limiting membrane.

Thus, Hedin (1) writes "da ferner der Zucker durch die
Membran nicht passieren kann, so übt der Zucker gegen die
Membran einen gewissen Druck aus. Dieser wird der *Os-
motische Druck* der eingeschlossenen Lösung genannt."

Wells (2) says "since osmotic pressure, exactly like gas pres-
sure, is presumably produced by the bombarding of the walls
of the container by particles in solution. . . . ."

Lewis (3) writes "It seems reasonable to suppose, therefore, that when diffusion of a solute does occur in a given direction it is due to the osmotic pressure acting as the driving force. Of course we cannot speak of the osmotic pressure of the solvent, but simply of the solute, since the concentration of the solute corresponds to gas concentration."

Nernst (4) writes "it must happen, of course, that the sugar will exert a *pressure* on the partition, which opposes its endeavor to fill the whole solution." The further exposition by this author leaves no doubt that he looks upon osmotic pressure as being directly exerted by the solute.

The second conception of the nature of the force of osmotic pressure is expressed by van't Hoff (5) as follows:

In order clearly to realize the quantity referred to as osmotic pressure, imagine a vessel completely full of an aqueous solution of sugar, placed in water. If it be conceived that the solid walls of this vessel are permeable to water but impermeable to the dissolved sugar, then, owing to the attraction of the solution for water, water will enter the vessel up to a certain limit, thereby increasing the pressure on the walls of the vessel [inside]. Equilibrium then ensues owing to the pressure resisting further entry of the water. This pressure we have termed osmotic pressure.

This conception ignores the dissolved substances as directly exerting the force that we are studying and looks upon the latter as merely the pressure developed by the accumulation of the water which diffuses through the semipermeable membrane into the solution containing the greater concentration of molecules and dissociated ions. The view just stated is not without adherents among the investigators of the subject of osmosis but it has received almost no consideration in the published treatises.

The preceding study of the boric acid hemolysis (6) had revealed conditions under which the development and effect of osmotic pressure can be exactly controlled and observed under varying quantitative relationships.

In previous investigations Eisenberg (7) had observed the laking of the corpuscles with formalin and urea under circumstances, which, so far as they were studied by him, coincide with those which we have found to control the phenomenon of boric acid hemolysis and we had observed a similar hemolytic action on the part of glycerin and ammonium chloride.

As will be presently set forth, a comparative study of the quantitative and time-relationships that govern the hemolytic effect of some of these reagents, has revealed facts which can be explained only with the assumption that *osmotic pressure is not a direct property of a solute but is developed indirectly, as a result of a process of diffusion, by the accumulation of water on one side of a semipermeable membrane.*

Eisenberg observed that corpuscles which had been in contact with formaldehyde in a concentration below that capable of "fixing" them were immediately laked on being suddenly immersed in isotonic saline solution. This hemolytic effect did not occur if the treated corpuscles were immersed in more concentrated salt solution. Eisenberg concluded that the formalin hemolysis is not a direct effect of the reagent. He considered the phenomenon as a "water hemolysis" but he adduced no experimental support for such assumption.

Eisenberg's observation of the urea hemolysis (in the lesser concentration of that substance in physiological saline solution) was confined to the mere fact that that effect is produced by a sudden immersion of the treated corpuscles in isotonic salt solution. Eisenberg excluded osmotic pressure as a cause of the urea hemolysis, because of the fact that with the lesser concentration of the reagent a longer contact was followed by a stronger hemolytic effect than a shorter contact. This fact, Eisenberg thought, pointed to a direct action of the urea.

The first experiments of the present study were designed for the purpose of determining whether the hemolytic action of formaldehyde and of urea were, like that of boric acid, the result of osmotic pressure and five criteria were used together in arriving at a conclusion as to that question. The first of these had already been applied by Eisenberg in his observation

that the hemolytic action of both substances was developed merely by a reduction in the concentration of the substance in the medium of suspension; the second criterion was the absence of hemolysis when the treated corpuscles were suddenly immersed in concentrated salt solutions; the third criterion was the disappearance of the peculiar sensitiveness of the treated corpuscles to immersion in physiological salt solution after being washed in concentrated salt solution; the fourth criterion was the absence of hemolysis when to the treated corpuscles a hemolytic volume of physiological salt solution was added not all at once, but gradually; and the fifth criterion was the demonstration of the permeability of the corpuscular membrane to both substances with the cryoscopic method of Hedin.

In all of these five respects the corpuscles treated with either formaldehyde or urea[1] behaved exactly like those treated with boric acid. Hence the conclusion is warranted that the hemolysis produced by the former two substances is, like that of boric acid, a result of osmotic pressure.

In order to make a comparative study of the osmotic hemolysis produced by the three selected reagents the concentrations were determined in which they all produce the same degree of hemolysis upon an arbitrarily selected, uniform diminution of those concentrations. It was found that if 0.05 cc. of corpuscular suspension were treated for ten minutes with 0.4 cc of 3.5 per cent boric acid or 0.4 cc. of 4 per cent formaldehyde or 0.8 cc. of 10 per cent urea, the total volume in each case being 1 cc., the sudden addition of 1 cc. of physiological salt solution would produce very strong hemolysis, while the addition of 2 cc. of that solution would cause complete hemolysis in all of the mixtures.

Under the condition of these comparative tests the assumption is justified that in all three instances, where the same degree of hemolysis was produced, the corpuscles were being subjected to the same degree of osmotic pressure.

[1]For a generous supply of urea of highest purity the author is indebted to Dr. William J. Gies of the College of Physicians and Surgeons in New York City.

On the basis of these tests, the same relative concentrations were used in the subsequent comparative study.

It was found that the osmotic concentrations of the mixtures containing 0.2 cc. of 3.5 per cent boric acid, or 0.2 cc. of 4 per cent formaldehyde or 0.4 cc. of 10 per cent urea were respectively Δ = 0.780, 1.091 and 1.91; and that the corresponding changes in concentration in the comparative hemolytic experiment were as follows:

	CONCENTRATION OF THE SUBSTANCE IN THE TREATING MIXTURE	CONCENTRATION OF THE SUBSTANCE IN THE MIXTURE AFTER ADDITION OF THE MINIMAL HEMOLYTIC AMOUNT OF PHYSIOLOGICAL SALT SOLUTION
	per cent	per cent
Boric acid, 3.5 per cent, 0.2 cc............	0.700	0.378
Formaldehyde, 4 per cent, 0.2 cc..........	0.800	0.267
Urea, 10 per cent, 0.4 cc.................	4.000	1.3

It is evident that if the destructive osmotic effect that we are studying is exerted directly by the molecules of the different substances, it should be expected that the solutions of the three substances which produce the same hemolytic effect would be found, by the cryoscopic method, to be of the same osmotic concentration; furthermore, it should be expected that a constant relation would be found between the concentration of each substance with which the corpuscles were treated and that of the respective mixture, after the minimal hemolytic quantity of physiological saline solution had been added.

However, in the experiments that were undertaken to determine this question, neither of these two requirements was satisfied.

It is seen that although the osmotic concentration of the mixture of boric acid used in the treatment of the corpuscles is considerably less than that of the other two substances the same hemolytic effect was produced by a change in the concentration of the reagent which was *much less* in the case of boric acid than it was in the other two substances; that is, *exactly the reverse* of what would have been expected if the osmotic force is exerted directly by the molecules of the reagent.

A comparison of the results obtained with formaldehyde and with urea shows a close correspondence in the ratios between the concentration used for treating the corpuscles and that resulting upon the addition of the minimal hemolytic amount of physiological saline solution. In view of the fact that the osmotic concentration of the mixture of formaldehyde used in treating the corpuscles was only about half as great as that of the treating mixture of urea a correspondingly *greater lowering of the concentration of* the former reagent should have

TABLE 1

Determination of the ratio of the final concentration to the original concentration of the reagent in boric acid hemolysis

	NUMBER OF TEST TUBE			
	I	II	III	IV
Boric acid solution (3.5 per cent) (cc.)...............	0.1	0.1	0.1	0.1
Physiological salt solution (cc.).....................	0.25	0.35	0.45	0.55
Blood suspension (cc.).............................	0.05	0.05	0.05	0.05
Whole volume (cc.)................................	0.4	0.5	0.6	0.7
Percentage concentration of boric acid in the treating mixtures...................................	0.875	0.7	0.583	0.5
Minimal hemolytic amount of physiological salt solution, added after five minutes contact without previous centrifugation (cc.).....................	0.4	0.65	0.9	1.4
Final percentage concentration of boric acid.........	0.438	0.304	0.233	0.167

been thought necessary to the production of an identical hemolytic effect in the two mixtures.

This latter contention is verified by an examination of the protocols of three series of tests that were carried out with diminishing concentrations of boric acid, formaldehyde and urea respectively. These protocols are presented in tables 1, 2 and 3.

In all of these three experiments it is evident that, as the concentration of the reagent that was used for treating the corpuscles diminishes, the ratio between that concentration and the final concentration rapidly increases.

TABLE 2

Determination of the ratio of the final concentration to the original concentration of the reagent in formaldehyde hemolysis

	NUMBER OF TEST TUBE			
	I	II	III	IV
Formaldehyde solution (4 per cent) (cc.)............	0.1	0.1	0.1	0.1
Physiological salt solution (cc.).....................	0.25	0.35	0.45	0.55
Blood suspension (cc.)...............................	0.05	0.05	0.05	0.05
Whole volume (cc.)	0.4	0.5	0.6	0.7
Percentage concentration of formaldehyde in the treating mixtures.................................	1.0	0.8	0.666	0.572
Minimal hemolytic amount of physiological salt solution, added after five minutes contact without previous centrifugation (cc.)......................	1.0	1.6	2.2	4.5
Final percentage concentration of formaldehyde.....	0.286	0.19	0.143	0.077

TABLE 3

Determination of the ratio of the final concentration to the original concentration of the reagent in urea hemolysis

	NUMBER OF TEST TUBE			
	I	II	III	IV
Urea solution (10 per cent) (cc.).....................	0.2	0.2	0.2	0.2
Physiological salt solution (cc.).....................	0.15	0.25	0.35	0.45
Blood suspension (cc.)...............................	0.05	0.05	0.05	0.05
Whole volume (cc.).................................	0.4	0.5	0.6	0.7
Percentage concentration of urea in the treating mixtures...	5.0	4.0	3.33	2.86
Minimal hemolytic amount of physiological salt solution, added after five minutes contact without previous centrifugation (cc.)..........................	0.85	1.3	2.8	9.0
Final percentage concentration of urea..............	1.6	1.11	0.59	0.206

The experimental facts thus far adduced in the comparative study of the osmotic hemolysis produced by the three selected reagents are incompatible with the theory that osmotic pressure is exerted directly by the molecules of the solute.

They demonstrate that in circumstances under which the three substances are allowed to act upon the corpuscular membrane in identical "osmotic concentration" the quantitative effect is by no means identical in the three instances. Under such circumstances the effect produced by boric acid is greater than that of formaldehyde and that of the latter substance is in turn, greater than that of urea.

These differences are made further apparent by an analysis of the results presented in tables 1, 2 and 3.

As the treating concentration of the three substances is diminished the ratio between that concentration and the final concentration (that is, the concentration of the substance in the mixture after the addition of the minimal completely hemolytic amount of physiological salt solution) increases much more rapidly in the case of urea than it does with formaldehyde and in the latter case more rapidly than it does with boric acid.

The respective ratios for the three substances are:

	RATIO OF FINAL CONCENTRATION TO TREATING CONCENTRATION	
	Highest treating concentration	Lowest treating concentration
Boric acid......................................	1–2	1–3
Formaldehyde..................................	1–3.5	1–7.4
Urea...	1–3.1	1–13.8

Differences in the osmotic effect of the three reagents corresponding with those already described were revealed in a comparative study of the quantitative relationships in the inhibition of the osmotic hemolysis with concentrated solutions of sodium chlorid and with solutions of the reagents themselves.

In these tests the treatment of the corpuscles and the determination of the minimal non-hemolytic concentrations of the various solutions was conducted in the manner of the tests

presented in table 7 of the previous paper. The osmotic concentrations tabulated under the heading of Δ were determined with the cryoscopic method.

The protocols of the tests are presented in tables 4, 5 and 6.

It is seen that when the corpuscles were treated with boric acid the osmotic concentrations of the minimal non-hemolytic concentrations of both sodium chlorid and boric acid are in each instance practically identical with the osmotic concentration of the treating mixture. This fact demonstrates that, given an equal intracorpuscular and extracorpuscular osmotic pressure, a

TABLE 4

Relation of the concentration of boric acid used in treatment to the minimal inhibiting concentrations of sodium chloride and boric acid

TREATING CONCENTRATION OF BORIC ACID			INHIBITING CONCENTRATION			
Amount of a 3.5 per cent solution in 1 cc. of original mixture	Per cent	Δ	NaCl		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ
cc.						
0.5	1.75	1.115	1.85	1.095	1.54	1.07
0.45	1.575	1.075	1.75	1.05	1.40	1.00
0.4	1.4	1.00	1.62	0.97	1.275	0.95
0.35	1.225	0.94	1.5	0.91	1.05	0.88
0.3	1.05	0.88	1.4	0.85	0.945	0.84
0.25	0.875	0.835	1.3	0.83	0.77	0.80
0.2	0.7·	0.78	1.18	0.74	0.595	0.75
0.15	0.525	0.73	1.08	0.69	0.42	0.67

slight lowering of the latter results in an appreciable hemolytic effect.

From the foregoing considerations it follows that if the assumption which we are examining is correct it should be expected that the hemolysis of the corpuscles which had been treated with any of the three different substances, in solutions of the same osmotic concentration, would be inhibited with the same minimal concentration of sodium chlorid.

Here again, however, the logical consequences of the theory were not fulfilled.

It is seen that when the osmotic concentration of the treating solution of boric acid was 1.00 the minimal inhibiting osmotic concentration of sodium chloride was 0.91, but when the corresponding osmotic concentration of formaldehyde (0.98) was used for treatment the minimal inhibiting osmotic concentration of sodium chlorid was only 0.747. Furthermore, it is seen that the minimal inhibiting osmotic concentration of sodium chlorid for corpuscles treated with urea in an osmotic concentration of 1.910 was less (1.00) than it was for corpuscles treated with formaldehyde of about the same osmotic concentration (1.868).

TABLE 5

Relation of the concentration of formaldehyde used in treatment to the minimal inhibiting concentrations of sodium chlorid, formaldehyde and boric acid

TREATING CONCENTRATION OF FORMALDEHYDE			INHIBITING CONCENTRATION					
Amount of a 4 per cent solution in 1 cc. of original mixture	Per cent	Δ	NaCl		Formaldehyde in physiological salt solution		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ	Per cent	Δ
cc.								
0.5	2.0	2.035	2.1	1.26	1.6	1.7		
0.45	1.8	1.868	2.0	1.242	1.4	1.55		
0.4	1.6	1.70	1.8	1.082	1.24	1.4		
0.35	1.4	1.55	1.7	1.0	1.16	1.35	1.47	1.03
0.3	1.2	1.40	1.6	0.95	1.0	1.244	1.225	0.94
0.25	1.0	1.244	1.5	0.91	0.72	1.05	1.40	0.905
0.2	0.8	1.091	1.3	0.83	0.56	0.93	0.945	0.84
0.15	0.6	0.98	1.2	0.747	0.4	0.867	0.77	0.8

Similar discrepancies are disclosed in the quantitative results obtained when the treated corpuscles were tested with solutions of the three hemolytic reagents. For example, when the corpuscles had been treated with urea the osmotic concentrations of the minimal non-hemolytic concentrations of boric acid were always practically the same as those of sodium chlorid, but these were less than the corresponding ones of formaldehyde, the latter in turn, being less than those of urea. These discrepancies increase as the treating concentration of the urea increases.

In order to obtain a clear understanding of the significance of the results obtained with the corpuscles treated with formaldehyde and urea it is necessary first to outline the consequences of the tests which must have been anticipated on the basis of the assumption that osmotic pressure is a direct property of the solute.

As has been shown, the blood corpuscular membrane is perfectly permeable to all of the three hemolytic substances that we are studying.

TABLE 6

Relation of the concentration of urea used in treatment to the minimal inhibiting concentrations of sodium chlorid, urea, formaldehyde and boric acid

TREATING CONCENTRATION OF UREA			INHIBITING CONCENTRATION							
Amount of a 10 per cent solution in 1 cc. of original mixture	Per cent	Δ	NaCl		Urea in physiological salt solution		Formaldehyde in physiological salt solution		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ	Per cent	Δ	Per cent	Δ
cc.										
0.5	5.0		1.95	1.19	3.5	1.78	1.4	1.55		
0.45	4.5		1.85	1.095	3.0	1.60	1.2	1.375		
0.4	4.0	1.91	1.7	1.0	2.5	1.40	1.0	1.244	1.47	1.03
0.35	3.5	1.78	1.6	0.95	2.25	1.35	0.8	1.091	1.225	0.94
0.3	3.0	1.60	1.45	0.90	1.8	1.2	0.6	0.95	1.085	0.905
0.25	2.5	1.40	1.35	0.835	1.5	1.05	0.4	0.869	0.77	0.8

It is evident, therefore, that, if osmotic pressure is directly exerted by the substance in solution, that pressure must be greatest at the instant of the sudden lowering of the extracorpuscular concentration, becoming rapidly less as more and more of the substance diffuses out of the cells into the medium of suspension.

Hence, in accordance with the theory that we are examining, a calculation of the counterpressure that should be expected to inhibit the osmotic hemolysis in any instance need take in consideration only by the original (treating) osmotic concentration of the hemolytic agent.

On the other hand, it is known that the corpuscular membrane is impermeable to sodium chlorid; hence the medium of suspension that contains only this substance should be considered as exerting a counter osmotic pressure which does not diminish but remains constant.

The incompatibility of the discrepancies which we have just been considering with the assumption that the solute exerts a direct osmotic pressure is the more clear, when it is recalled that in the case of boric acid only a slight lowering of the extracorpuscular concentration of that substance is sufficient to cause a perceptible hemolysis of the treated corpuscles. This observation stands in striking and significant contrast with the considerable lowering of the extracorpuscular concentration of urea which is necessary for the development of the slightest degree of destructive osmotic pressure by that substance.

This contrast is consistent with that already noted between the respective ratios of treating and final concentrations when the minimal completely hemolytic amount of physiological saline solution is added.

Reviewing the foregoing experiments we find:

1. That the same degree of osmotic hemolysis is not produced by identical "osmotic concentrations" of boric acid, formaldehyde and urea nor by a corresponding lowering in the concentration of the substances in the medium of suspension of the treated corpuscles.

2. That as the treating concentration of the three hemolytic substances is correspondingly diminished the ratio between that concentration and the final concentration, in the hemolytic experiment, increases disproportionately with the different substances.

3. That the osmotic hemolysis of corpuscles which have been treated with the three hemolytic substances in the same "osmotic concentration" is not inhibited by identical concentrations of sodium chloride nor of the hemolytic substances themselves.

4. That all of these facts contradict the assumption that osmotic pressure is exerted directly by the solute.

It will be seen that the obstacles presented by the foregoing experimental results to the acceptance of the theory of osmotic pressure which we have been considering, offer no hindrance to the adoption of the alternative view.

According to the latter conception the destructive force developed in osmotic hemolysis is exerted, not by the hemolytic substance itself, but by the water that diffuses *into* the corpuscles under the influence of the *higher concentration* of the hemolytic substance *within* the cells.

Under this conception another factor, in addition to that of the concentration of the hemolytic agent, must be taken into account; namely, the factor of *time*. The diffusion of water through a semipermeable membrane into a concentrated solution takes place not merely in the first moment of the process of osmosis but over a period of time.

Hence, it is evident that in the case of a diffusible solute, such as the three hemolytic agents employed above, the degree of destructive pressure developed within the corpuscles, under the alternative theory, need not be determined solely by the *original concentration* of the substance in the cells. The degree of that pressure could conceivably be dependent on the *length of time* during which an effective inequality of concentration within and without the cells is maintained.

In other words if one of the hemolytic substances diffuses out of the corpuscles more rapidly than the others the higher intracorpuscular concentration of that substance (upon which the inward diffusion of water depends) is consequently maintained for a shorter time than in the latter two cases and the resulting intracorpuscular pressure must, therefore, be less. Hence in order to explain the foregoing quantitative differences in the hemolytic effect of identical "osmotic concentrations" of the three hemolytic agents it is necessary only to assume that formaldehyde diffuses through the corpuscular membrane more rapidly than does boric acid and less rapidly than does urea.

For example, if formaldehyde diffuses out of the corpuscles more rapidly than does boric acid then a higher original "os-

motic concentration" of the former substance must be used in order to maintain an effective intracorpuscular concentration over the *period of time* necessary for the inward diffusion of a *hemolytic volume of water*. A similar explanation is applicable to the quantitative differences observed between the hemolytic action of formaldehyde and of urea.

The application of this explanation to the other consistent differences in the behaviour of the three hemolytic substances that have been described above is too obvious to require detailed analysis.

There remains, however, to subject the assumption on which the explanation is based to experimental examination. In other words, it has yet to be shown that actually formaldehyde diffuses through the corpuscular membrane more rapidly than boric acid and less rapidly than urea.

This question was investigated in the following manner:

The unit of corpuscles was suspended in 1 cc. of solutions of three reagents in such concentrations that in each case, after an equality of concentration inside and outside the corpuscles had been established the addition of 1 cc. of physiological salt solution would cause very strong hemolysis and the addition of 2 cc. would cause complete hemolysis. With the use of numerous identical mixtures tests could be made at different intervals of time, so that the length of time required for the equalization of the intracorpuscular and extracorpuscular concentration; that is, for the whole process of diffusion of the substance into the corpuscles, could be determined for each reagent.

The protocol of this experiment is presented in table 7.

It is seen that under the conditions of the test the diffusion time of boric acid was ninety seconds, that of formaldehyde was thirty seconds, while that of urea was too short—less than five seconds—to be accurately measured.

The results of this experiment, thus, confirm our assumption and support the conclusion that osmotic pressure is not a direct property of a solute but is solely the pressure exerted by water which has passed, by the yet unexplained process of diffusion,

TABLE 7

Determination of the diffusion time of the three hemolytic substances

Treating mixtures

	cc.
Boric acid (3.5 per cent)...	0.4
Physiological salt solution.......................................	0.55
Blood suspension..	0.05

Formaldehyde (4 per cent).......................................	0.4
Physiological salt solution.......................................	0.55
Blood suspension..	0.05

Urea (10 per cent)..	0.8
Physiological salt solution.......................................	0.15
Blood suspension..	0.05

	PERIOD OF CONTACT	HEMOLYSIS AFTER THE ADDITION OF PHYSIOLOGICAL SALT SOLUTION	
		1 cc.	2 cc.
Boric acid.................................	5 seconds	0	0
	15 seconds	tr	w
	30 seconds	w	st
	1 minute	st	al.c
	1.5 minutes	v.st	c
	7 minutes	v.st	c
Formaldehyde...........................	5 seconds	tr	w
	15 seconds	w	v.st
	30 seconds	v.st	c
	1 minute	v.st	c
	7 minutes	v.st	c
Urea....................................	5 seconds	v.st	c
	10 seconds	v.st	c
	1 minute	v.st	c
	7 minutes	v.st	c -

through a semipermeable membrane to the side of the higher osmotic concentration.

In further elucidation of this conception it may be said that there appears to be no more reason to assume that the water which diffuses into the corpuscles exerts an inward pressure during that process than there is for the now intenable theory of a direct outward osmotic pressure on the part of the solute. Such an assumption would leave without explanation the resulting hemolysis, which is caused by the increased internal pressure and which could not occur if, as is thought by some, the water diffuses through the membrane *under pressure* and if such diffusion ceases only when the internal pressure becomes so great as to equalize the inward pressure of the entering water.

ADDENDUM

In view of the differences in the rate of diffusion of the three hemolytic agents it could be anticipated that when corpuscles treated with a substance of a lesser rate of diffusion are placed in solutions of a substance of a higher rate of diffusion the equalization of the intra and extra-corpuscular concentration of the latter substance would be accomplished before all of the substance, used in treating the corpuscles, had diffused out of them. Under these circumstances a certain degree of hemolysis should, therefore, be expected, and the experiment conducted in this manner confirmed the anticipation.

The protocol of this experiment is presented in table 8.

It is seen that in every case the solutions of a substance of lower diffusion rate inhibited the hemolysis of corpuscles treated with a substance of higher diffusion rate, but that, on the other hand, solutions of a substance of higher diffusion rate could not inhibit the hemolysis of corpuscles treated with a substance of lower diffusion rate. It was found, furthermore, that no concentration of urea was able to inhibit, in the least degree, the hemolysis of corpuscles treated with boric acid.

In conclusion I wish to express my indebtedness to Dr. A. F. Coca for his kind advice and direction in carrying out these experiments.

TABLE 8

Showing the inhibiting effect of "iso-osmotic" solutions of boric acid, formaldehyde and urea upon the hemolysis of corpuscles treated with those substances

Treating mixtures

cc.

Boric acid solution (3.5 per cent)................................. 0.5
Physiological salt solutions...................................... 0.45
Blood suspension... 0.05

Formaldehyde solution (4.0 per cent)....:....................... 0.5
Physiological salt solution...................................... 0.45
Blood suspension... 0.05

Urea solution (10 per cent)..................................... 0.75
Physiological salt solution 0.2
Blood suspension... 0.05

TREATING	ADDING (1 cc.)			
	1.8 per cent solution of ClNa $\Delta=1.082$	Boric acid in physiological salt solution, 1.56 per cent, $\Delta=1.078$	Formaldeyde in physiological salt solution, 0.8 per cent, $\Delta=1.091$	Urea in physiological salt solution, 1.6 per cent $\Delta=1.09$
Boric acid.........	0		st	c
Formaldehyde.......	0	0		st
Urea...............	0	0	0	

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STUDIES IN SPECIFIC HYPERSENSITIVENESS[1]

I. THE DIAGNOSTIC CUTANEOUS REACTION IN ALLERGY. COMPARISON OF THE INTRADERMAL METHOD (COOKE) AND THE SCRATCH METHOD (SCHLOSS)

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The first recorded experimental observation of cutaneous sensitiveness in allergy, is found in the classic monograph of Blackley (1) in which the causal relationship of the pollens to seasonal hay fever was demonstrated.

Blackley, on several occasions applied some pollen to the surface of the skin, which had been abraded as for small pox vaccination and each time, he obtained a deep and extensive swelling of the skin.

In a case of hypersensitiveness to buckwheat, reported by H. L. Smith (11) in 1909, and supposed by that author to be a case of "fagopyrismus," an experiment similar to that of Blackley was carried out by Rufus Cole. Cole used a sterilized infusion of buckwheat which he rubbed into a scarified area of the skin. This treatment resulted in the formation of a wheal of the size of a silver half-dollar and in the development of severe constitutional symptoms.

In the following year, Schmidt (10) reported a series of observations on the reactivity of the skin to Puro,[2] and a trypsin digested milk. The purpose of Schmidt's investigation was to search for any possible differences in the cutaneous reaction to these materials. In this main idea he failed, probably because no suitable cases of hypersensitiveness to the substances in his preparations was encountered.

Schmidt adopted the von Pirquet technic. The reactions that he did observe reached their greatest intensity after sixteen to eighteen hours; they were thus quite different from those elicited in allergy, which, with the von Pirquet technic are at their height within thirty minutes.

After Cole's experiment, the first successful attempt to make diagnostic use of the skin reactivity in allergy was reported by Oscar M. Schloss (8) in 1912 in a case of allergy to egg, almond and oat. Schloss used the von Pirquet borer for applying the tests, which were made with isolated proteins of eggwhite and preparations from the other two foods.

There is no doubt that this well conceived pioneer work of Schloss inspired much of the subsequent investigation of others, yet acknowledgement of his service is omitted in most of the literature of the subject.

Goodale (6), in 1916 wrote, "It has been known for some years that an individual sensitized to a given proteid may exhibit a characteristic reaction if the proteid is brought in a soluble form in sufficient concentration into contact with a scratch in the skin."

I. Chandler Walker (13) in 1917 wrote, "It has been known for some years that an individual sensitized to a given proteid may exhibit a characteristic reaction if the proteid is brought in a soluble form in sufficient concentration into contact with a scratch in the skin."

In all of Walker's investigations, the skin tests have been made according to the Schloss technic, and since 1916, the test

[2] A commercial preparation composed of meat extract, egg white, glycerin and salts.

material has usually been applied in the form of a powder representing the dry residue of an extract of the original material.

The method of preparation of the test substance is found in a paper by Wodehouse (17).   The material is extracted with water and the extract is dried with the use of an electric fan until it becomes of a syrupy consistency.   To this fluid are added three to four volumes of 95 per cent alcohol and the resultant precipitate is washed with alcohol 95 per cent, absolute alcohol and ether.

Talbot (12) referred to the "brilliant investigations of Oscar M. Schloss."   This author applied the skin test according to Schloss.

In 1910 Moss (7) urged the use of a cutaneous test before the reinjection of antitoxic serum and in the test he employed an intradermal injection method injecting 0.01 cc. of undiluted serum.   The reactions, which he observed, occurred at considerable intervals after the injections (ten to twenty-four hours) and were, therefore, different from the immediate reactions seen in the natural allergies.   On account of the limited scope of this investigation, the technic adopted by Moss did not become generally known nor used.

In 1911, Robert A. Cooke (3, 4), began his independent studies in allergy.   From the beginning Cooke's chief purpose was the application of the principle of desensitization in the prevention and treatment of the symptoms of allergy and as this involved the injection of the substance, it was convenient to use the latter in liquid form.   It was convenient, also, in using the liquid preparations for the skin test, to apply them by the intradermal or injection method, and this method was adopted by Cooke and employed by him and his assistants and pupils to the present time.   This method is the same as that used afterwards by Schick in his well known test of immunity to diphtheria.

Thus there are in use at present two different methods of testing the skin in allergic conditions, the method of Schloss, which we shall refer to as the cutaneous or scratch test and the method of Cooke, which may be designated as the intradermal or injection test.

Regarding the relative efficiency of the two methods Schloss (9) expresses the opinion that the intradermal test is more sensitive than the cutaneous, but thinks the former is "apt to be misleading. Pseudo-reactions occur which are difficult to interpret, ... On two occasions I have seen severe infections due to such tests."

I. C. Walker and J. Adkinson (16) have attempted a comparison of the two methods. From the published report of their experiments it is evident that these authors were seriously handicapped by a lack of acquaintance with the proper method of applying the intradermal test and by inexperience in interpreting the reactions produced by this test. They injected 0.1 cc., which is five to ten times the volume of fluid necessary for this mode of application and they arbitrarily considered that "any appreciable increase over these normal measurements (of the swelling produced mechanically by this large injection) means a positive reaction."

This unfortunate circumstance vitiates all of their comparative experiments and consequently nullifies their conclusions. Incidentally, one of these conclusions is that the intradermal test is "much too sensitive, therefore it is not an index to proper treatment." In this connection it may be observed; first, that there is no need for such an index; secondly, that the intradermal reaction properly applied is distinctly modified by specific treatment in a way that could be used if it were necessary and thirdly, that the advantage claimed for the scratch test by the authors mentioned depends, as we shall show, upon a serious defect in that test—a defect evidenced by the fact that it produces a positive reaction only in the more sensitive individuals. In other words, after treatment has been carried to a certain point the scratch test is no longer able to produce a positive reaction. In the present communication it is intended to report the results of comparative skin tests carried out on the same individuals with the two methods, and as different materials were used, the investigation also compared the dry extracts with the fluid preparations.

TECHNIC

The intradermal or injection test

The fluid preparations used for these tests were obtained from
the Department of Applied Immunology in the New York
Hospital. The methods of preparing these materials are de-
scribed elsewhere in this issue (2). The fluid preparations of
horse dander contained 0.04 mgm. of protein nitrogen per
cubic centimeter; those of the other materials contained 0.5
miligrams of nitrogen per cubic centimeter.

The injection of the fluid was made with the tuberculin syringe,
which is graduated in hundredths of a cubic centimeter. The
hypodermic needles were of 26 gauge. The syringe and needles
were sterilized by boiling.

The injection was made into the skin on the outer aspect
of the upper arm. About 0.01 cc. was injected, forming an eleva-
tion about 2 to 3 mm. in diameter. Previous to the injection the
skin was cleansed with alcohol.

The results of the intradermal tests were noted five to ten
minutes after the injection, the height of the reaction being
reached within that time.

The cutaneous or scratch test

These tests were carried out with two different kinds of prep-
arations. One was in the form of a powder supplied by a
commercial organization and obtained in the open market.
The other was the same as those used for the intradermal tests.

The dry preparations were used as follows: An abrasion or
scratch ⅛ inch (3 mm.) long was made with a von Pirquet borer,
on the flexor surface of the forearm. The abrasion was not made
deep enough to draw blood. A small quantity of the powder was
applied to the abrasion, moistened with a drop of decinormal
sodium hydrate solution and rubbed into the scratch. For the
purpose of control a similar abrasion was made into which only
the decinormal alkali was rubbed. After twenty to thirty
minutes the test mixture was washed off with 50 per cent
alcohol and the result was noted.

When the scratch test was carried out with the fluid preparations, a drop of the latter was rubbed into the abrasion, without addition of sodium hydrate. In these cases the control tests were made with sterile normal salt solution. The results of these tests were noted after five to twenty minutes.

A positive reaction in both tests was determined by the formation of a wheal with an area of erythema surrounding it. To be of diagnostic significance the wheal must be at least 0.5 cm. in diameter. If the wheal was 1–2 cm. in diameter the reaction was given the designation of ++; if the diameter of the wheal exceeded 2 cm. the reaction was designated +++. In the larger reactions irregularity of outline of the wheal was noted and "pseudopod" formation, which was more common and more marked with the intradermal test.

The comparative tests were carried out with the following preparations:

Animal emanations. Horse, dog, cat, rabbit, goose.

Foods. Egg white, wheat, milk albumin, casein.

The individuals upon whom the comparative tests were performed were all subject to asthma.

The causal relationship of the respective material to the asthmatic symptoms in each case was established on two or more grounds, which are enumerated below and designated in the tables by the corresponding numbers.

1. Symptoms appear after contact with or upon proximity to the original material.

2. Asthmatic symptoms develop after a diagnostic injection.

3. Asthmatic symptoms disappear after a course of therapeutic injections.

4. Symptoms disappear upon avoidance of or removal of the original material.

It will be seen that in every case the intradermal test with the respective protein resulted positively. Objection may be made to our selection of cases on the ground that it was made with reference to the result of the intradermal test. We can only reply to such objections, that with the most conscientious observation of the cases presenting themselves, we have encountered

among those clinically sensitive to the proteins used in this comparative study, none in which the intradermal tests with the respective protein resulted negatively. Our experience with most fruit juices has been different from this. With these preparations, we have observed some negative reactions in individuals that were clearly sensitive to the original material.

The results of the comparative tests are presented in tables 1 to 6.

TABLE 1

NUMBER	CASE	Commercial	Fluid preparations (Coca)		RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Horse dander	Horse epithelium		
		Scratch test	Intradermal	Scratch	
1	2686 C	+	++	Not done	1, 3 and 4
2	61181c	+	+++	+	1 and 4
3	67090c	++	+++	++	1, 3 and 4
4	67250c	+	+++	++	1 and 4
5	65423c	++	++	++	1, 3 and 4
6	67602c	+	+++	++	1 and 4
7	68146c	++	+++	++	1, 3 and 4
8	66140c	+	+++	++	1, 3 and 4
9	70446c	+	+++	++	1 and 4
10	69670c	+	+++	++	1 and 4
11	2711 C	−	+++	++	1, 3 and 4
12	581 C	−	+++	++	1, 3 and 4
13	67351c	−	+++	++	1, 3 and 4
14	67787c	−	+++	++	1, 3 and 4
15	66767c	−	+++	++	1 and 4
Summary	15	Positive 10 Negative 5	Positive 15	Positive 14	

The individual tables need no explanatory comment. The results in each series were concordant in the sense that:

1. The intradermal test is superior to the cutaneous or scratch test.

2. The fluid preparations made according to the methods of Coca are superior to the corresponding dry commercial preparations.

TABLE 2

NUMBER	CASE	Commercial powder	Fluid preparations (Coca)		RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Dog hair	Dog epithelium		
		Scratch test	Intradermal	Scratch	
1	2686 C	+	+++	+++	1 and 4
2	61181c	+	+++	++	1 and 4
3	67090c	++	+++	++	1 and 4
4	65423c	+	+++	++	1 and 4
5	66804c	+	++	++	1 and 4
6	69670c	+	+++	++	1 and 4
7	2691 C	−	+++	Not done	1, 3 and 4
8	67351c	−	+++	++	1 and 4
9	2782 C	−	+	+	1 and 4
10	18B	−	+++	−	1 and 4
11	67096c	−	+++	−	1 and 4
12	61B	−	+++	++	1, 3 and 4
Summary	12	Positive 6 Negative 6	Positive 12 Negative 0	Positive 9 Negative 2	

ANIMAL EMANATIONS (spanning header over Commercial powder / Fluid preparations)

TABLE 3

NUMBER	CASE	Commercial Rabbit hair powder	Fluid preparations (Coca)		RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
			Rabbit epithelium		
		Scratch test	Intradermal	Scratch	
1	67250c	+++	+++	++	1 and 4
2	66761c	+	++	++	1 and 4
3	66769c	+	++	+	1 and 4
4	68146c	++	+++	++	1 and 4
5	66804c	++	+++	++	1 and 4
6	63B	+	+++	+	1 and 4
7	2713C	−	+++	+	1 and 4
8	69670c	−	+++	−	1 and 4
9	48B	−	+++	−	1, 3 and 4
Summary	0	Positive 6 Negative 3	Positive 9 Negative 0	Positive 7 Negative 2	

ANIMAL EMANATIONS (spanning header over Commercial / Fluid preparations)

TABLE 4

NUMBER	CASE	ANIMAL EMANATIONS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Goose feathers powder	Goose epithelium		
		Scratch test	Intradermal	Scratch	
1	2713 C	++	+++	Not done	1 and 4
2	61181c	++	+++	++	1, 3 and 4
3	67090c	++	+++	++	1 and 4
4	67250c	++	+++	++	1 and 4
5	66804c	+	++	++	1 and 4
6	2648 C	−	+++	Not done	1 and 4
7	1949 C	−	+++	Not done	1 and 4
8	67351c	−	+++	++	1 and 4
9	66769c	−	+++	++	1 and 4
10	67602c	−	+++	++	1 and 4
11	67787c	−	+++	+	1 and 4
12	2782 C	−	+	++	1 and 4
13	68146c	−	+++	+	1 and 4
14	66146c	−	+++	+	1 and 4
Summary	14	Positive 5 Negative 9	Positive 14 Negative 0	Positive 11 Negative 0	

TABLE 5

NUMBER	CASE	ANIMAL EMANATIONS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Cat hair powder	Cat epithelium		
		Scratch test	Intradermal	Scratch	
1	2739 C	+	+++	+	1, 3 and 4
		−	+++	+	1, 3 and 4*
2	61181c	++	+++	+	1 and 4
3	67090c	+	+++	++	1 and 4
4	66761c	+	+++	++	1 and 4
5	67351c	+	+++	++	1 and 4
6	66995c	+	+++	++	1 and 4
7	67787c	+	+	+	1 and 4
8	2782 C	+	+++	++	1 and 4
9	70446c	+	+++	++	1 and 4
10	2601 C	−	+++	Not done	1 and 4
11	2711 C	−	+++	−	1, 3 and 4
		−	+++	−	1, 3 and 4*
12	2713 C	−	+++	−	1, 3 and 4
13	60670c	−	+++	++	1 and 4
14	15B	−	+++	++	1, 2, 3, and 4
15	67096c	−	+++	−	1 and 4
Summary	17	Positive 9 Negative 8	Positive 17 Negative 0	Positive 12 Negative 4	

* Test 2 weeks later.

TABLE 6

NUMBER	CASE	FOOD PROTEINS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Egg white powder	Egg white		
		Scratch test	Intradermal	Scratch	
1	1949C	++	+++	Not done	1 and 4
2	66360c	+++	+++	++	1 and 4
3	2671C	+	+++	Not done	1 and 4
4	2699c	−	+++	Not done	1 and 4

Wheat

5	70248c	−	+	+	1, 3 and 4
		−	+	−	1, 3 and 4*
6	69839c	−	+++	++	1, 3 and 4
7	70831c	−	+++	−	1, 3 and 4
8	61B	−	+++	−	1 and 4

Milk albumin

| 9 | 1949c | − | +++ | Not done | 1 and 4 |

Casein

| 10 | 1949c | − | +++ | Not done | 1 and 4 |
| Summary | 11 | Positive 3 Negative 8 | Positive 11 Negative 0 | Positive 3 Negative 4 | |

* Test 2 weeks later.

SUMMARY TABLES 1-6

	COMMERCIAL POWDERS SCRATCH METHOD				FLUID PREPARATIONS INTRADERMAL METHOD				FLUID PREPARATIONS SCRATCH METHOD			
	−	+	++	+++	−	+	++	+++	−	+	++	+++
1	5	7	3	0	0	0	2	13	0	1	13	0
2	6	5	1	0	0	1	1	10	2	1	7	1
3	3	3	2	1	0	0	2	7	2	3	4	0
4	9	1	4	0	0	1	1	12	0	3	8	0
5	8	8	1	0	0	1	0	16	4	4	8	0
6	8	1	1	1	0	2	0	9	4	1	2	0
Summary	39	25	12	2		5	6	67	12	13	42	1

The first conclusion is drawn from a comparison of the middle and right hand columns of the summary of the tables.   It is seen that when fluid preparations were used, the reaction was positive in all of the 78 cases with the intradermal method, but negative in 12 of the cases with the scratch method.

The second conclusion is drawn from a comparison of the left hand and right hand columns of that summary.   When the scratch method was used with the fluid preparations the reaction was negative in 12; that is, about 18 per cent of the cases tested. With the commercial powders the scratch method resulted negatively in 39 cases or 50 per cent of those tested.

The two methods of applying the skin tests must be compared in other respects besides these that we have already discussed.

1. Relative ease of performing the tests

The scratch method has been preferred by some because it was thought to be more easily applied than the intradermal method.   Our own experience with the two procedures has satisfied us that the reverse is true.

As it is often necessary to carry out the skin tests with a number of different substances as well as with different dilutions of the same substance, the time required for the test is a practically important consideration, and moreover the time factor may be taken as a fair index of the relative ease with which the two methods of testing can be applied.

We have carried out a series of 52 parallel tests in the same individuals, with the purpose of determining the relative time required for the application of the two methods.   The fluid preparations of Coca were employed for both methods.   This was done for the following reasons.

The fluid preparations can be applied more quickly with the scratch technic, than the dry products and the reaction with the fluid preparations reaches its maximum sooner than that with the dry products.   Moreover, as we have shown above, the cutaneous reaction is generally more marked with the fluid preparations, than with the dry ones.   The data obtained from these parallel tests are presented in table 7.

The results of these comparative tests demonstrate that even with the more convenient and more active fluid preparations over 50 per cent more time is required for the development of a positive reaction with the scratch technic than is needed with the injection method.

TABLE 7

REACTION TIME WITH THE INTRADERMAL METHOD		REACTION TIME WITH THE SCRATCH METHOD	
10 minutes	7 minutes	10 minutes	14 minutes
8 minutes	10 minutes	11 minutes	9 minutes
10 minutes	6 minutes	10 minutes	13 minutes
· 10 minutes	10 minutes	10 minutes	10 minutes
7 minutes	5 minutes	10 minutes	9 minutes
10 minutes	5 minutes	7 minutes	17 minutes
7 minutes	10 minutes	15 minutes	14 minutes
11 minutes	7 minutes	8 minutes	7 minutes
5 minutes	6 minutes	13 minutes	9 minutes
10 minutes	4 minutes	7 minutes	7 minutes
5 minutes	9 minutes	14 minutes	18 minutes
7 minutes	10 minutes	7 minutes	10 minutes
7 minutes	5 minutes	14 minutes	8 minutes
5 minutes	8 minutes	15 minutes	17 minutes
4 minutes	4 minutes	22 minutes	10 minutes
11 minutes	9 minutes	10 minutes	13 minutes
10 minutes	6 minutes	10 minutes	11 minutes
6 minutes	5 minutes	7 minutes	8 minutes
5 minutes	8 minutes·	12 minutes	11 minutes
10 minutes	4 minutes	14 minutes	10 minutes
10 minutes	5 minutes	5 minutes	12 minutes
5 minutes	5 minutes	12 minutes	14 minutes
10 minutes	5 minutes	10 minutes	11 minutes
6 minutes	6 minutes	7 minutes	8 minutes
5 minutes	7 minutes	17 minutes	13 minutes
9 minutes	8 minutes	12 minutes	9 minutes
Average reaction in 7$\frac{1}{4}$ minutes		Average reaction in 11$\frac{2}{3}$ minutes	
Earliest reaction in 4 minutes		Earliest reaction in 5 minutes	
Latest reaction in 11 minutes		Latest reaction in 22 minutes	

Less time is needed also in applying the intradermal test, than is required for the scratch test. With an adequate supply of sterile syringes 20 intradermal tests can be made in five minutes. With the dry commercial preparations the 20 tests can hardly be made in less than thirty minutes.

It is advised by the manufacturers of the dry preparations to make the "readings" after thirty minutes. In a large clinic or in an active private office, the shorter time needed for applying and reading the intradermal tests, weighs heavily in favor of this method.

2. Relative convenience to the patient

With the intradermal method a minimal amount of damage of damage is inflicted on the skin—merely the prick of a sharp, fine hypodermic needle. With the scratch method an incision ¼ inch long is made or an area of equal diameter is abraded by scraping. The rubbing of the test mixture into the traumatized skin does further damage. We believe that the intradermal method is the less painful of the two. The marks of the injection method usually disappear within a week; those resulting from the scratch method often persist for weeks or even for months.

3. Greater convenience of the fluid preparations

Considerable convenience is afforded in the use of the fluid extracts by the fact that the same preparation is used for diagnosis and for treatment. This advantage of the fluid extracts over the dry preparations becomes more important as the number of preparations used increases.

4. Constitutional reactions

All other factors being equal, the quantity of material absorbed after its injection should be greater than after its application to an abraded surface. Hence it should be expected that constitutional reactions would be more frequent with the intradermal method than with the scratch method. The writer is unable to say whether this is actually true. The experiences of Rufus Cole cited above and of Cooke (5) demonstrate that constitutional reactions may result from either the scratch method or the intradermal method.

With regard to the possibility of infection resulting from the intradermal injection, which was mentioned by Schloss, we can only say that with ordinary aseptic precautions in the preparation of the extracts and in their use, there is no danger of infection. In the writer's experience, which is in agreement with that of Cooke and that of Vander Veer, infection would occur only as a result of gross disregard of the simple principles of ordinary asepsis.

SUMMARY AND CONCLUSIONS

Two methods of applying the skin test in allergic conditions are in use:

1. The cutaneous or scratch method of Schloss.
2. The intradermal or injection method of Cooke.

Two forms of test proteins are in use:

1. The dry powdered preparations made according to the methods described by Wodehouse.
2. The fluid preparations originally used by Cooke and made now according to the methods described by Coca.

In a series of 78 comparative tests the superiority of the intradermal method over the scratch method has been shown upon the following grounds:

1. In every case known to be clinically sensitive to a protein, the intradermal test with that protein resulted positively. The scratch test with the corresponding dry preparations resulted positively in only half the cases tested. The scratch test with the fluid preparations resulted negatively in 18 per cent of the cases tested.

2. The intradermal method properly applied is not so painful as the scratch method and the resulting markings of the skin do not persist so long after the former method.

3. Less time is required for applying the intradermal method and for obtaining the results than is needed for the scratch method.

4. The same preparation can be used for testing and for treatment when the fluid preparations are employed.

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

II. A COMPARISON OF VARIOUS POLLEN EXTRACTS WITH REFERENCE TO THE QUESTION OF THEIR THERAPEUTIC VALUE IN HAY FEVER

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The following study of the relative strengths of various commercial extracts used in the treatment of hay fever was made because there is no recognized standard method of preparation of such extracts and it was felt that it might be of interest to see what therapeutic results might reasonably be expected from their use. Preparations of four of the best known commercial products were purchased from a pharmacy and compared with corresponding extracts obtained from the Department of Applied Immunology in the New York Hospital. The method of preparation of these latter extracts is published by Dr. A. F. Coca in this issue of the Journal of Immunology (1). The commercial products have been designated A, B, C, and D, while those made in the New York Hospital are labelled E. For convenience the last named extracts will be referred to as the "Cornell preparations." All of the five preparations were in fluid form. Three strengths (designated 1, 2 and 3, in order of strength) were tested of preparation A, three of B, two of C, two of D and three of E. Preliminary tests showed that only the very sensitive cases gave any ophthalmic reactions to the preparations A, B, C and D, hence our study was necessarily limited to a relatively small group. Eighteen cases in all were used for this purpose but they were not all tested for each preparation and each strength, since it is not practicable to test with more than three or four extracts at

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TABLE 1

History Number	A 1 skin	A 1 eye	A 2 skin	A 2 eye	A 3 skin	A 3 eye	B 1 skin	B 1 eye	B 2 skin	B 2 eye	B 3 skin	B 3 eye	C 1 skin	C 1 eye	C 2 skin	C 2 eye	D 1 skin	D 1 eye	D 2 skin	D 2 eye	E 1 skin	E 1 eye	E 2 skin	E 2 eye	E 3 skin	E 3 eye
2581																										
3193																										
3166																										
3144																										
3207																										
2001																										
3208																										
3121																										
3110																										
2584																										
1156																										
3245																										
2965																										
2273																										
1550																										
2900																										
1409																										
158																										

one sitting. This is particularly true if a positive ophthalmic reaction is obtained with one of the first extracts. In this event, no more tests can be made in this eye at that time. As the ophthalmic reaction often becomes less pronounced after a few therapeutic injections the same patient cannot be tested with other extracts at a later date for comparison, unless it is determined that such a diminution in sensitiveness has not taken place. This could often be determined by testing one eye with the same strength of the Cornell preparation which had given a reaction on a previous testing. If the reaction was the same it was fair
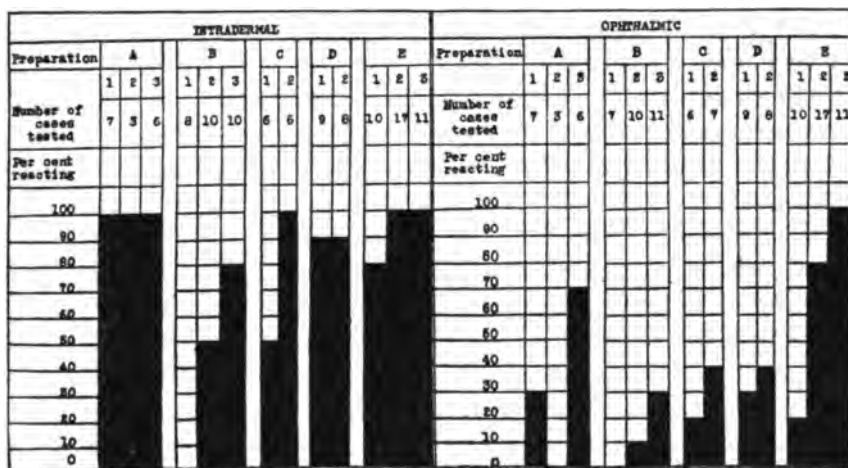
CHART 1

to assume that no diminution in sensitiveness had taken place and the other eye was then used for further testing. All of the eighteen cases were tested with one or more of the Cornell preparations, while from six to ten cases were tested with each of the other extracts (with the exception of preparation A2 to be mentioned below). The results are tabulated in table 1 and a graphic representation of these results is given in chart 1. The following symbols are used to designate reactions; 0 = negative; + = slight; ++ = moderate; +++ = marked. In the graphic chart 0 and + are considered negative reactions, while ++ and +++ are positive reactions for the intradermal tests. For the

ophthalmic tests, 0 indicates a negative and $+$, $++$, and $+++$ indicate positive reactions.

The strength of the various preparations is said by the producers to be as follows:

A1 = 1000 pollen units; A2 = 1250 pollen units; A3 = 1500 pollen units.

B1 = 10 pollen units; B2 = 100 pollen units; B3 = 1000 pollen units.

C1 = 0.02 mgm. of nitrogen to 1 cc.; C2 = 0.08 mgm. of nitrogen to 1 cc.

D1 = 1:1000 dilution; D2 = 1:100 dilution.

E1 = 0.001 mgm. of nitrogen to 1 cc., E2 = 0.005 mgm., E3 = 0.01 mgm. of nitrogen to 1 cc.

The methods of determining the value of the pollen units in preparations A and B; of determining the nitrogen in preparation C and of making the dilution in preparation D are not known to the author. The nitrogen in preparation E was determined by the Kjeldahl method.

In comparing the results of the tests both the intradermal and the ophthalmic reactions were considered but as the eye is less sensitive than the skin (by the intradermal test) and therefore requires a stronger solution to give a positive reaction the difference in the strengths of the solutions is more clearly demonstrated in the chart of the ophthalmic reactions. A résumé of these results shows that (with one exception to be noted later) rough comparisons may be drawn between A, B, C, and D, with E as follows: The activity of preparation A1 is equal to that of preparation E1; the result with preparation A2 must be disregarded because only three cases were tested with this extract and, while these all gave positive intradermal reactions, they all gave negative ophthalmic reactions; (it happened that among the group of eighteen tested these three individuals were comparatively insensitive and it is probable that if five or six more cases had been tested enough of them would have reacted with A2 to give a higher percentage of ophthalmic reactions); the activity of preparation A3 is equal to that of preparation E2; preparation B1 was apparently inert as it produced no intrader-

mal or ophthalmic reaction in the eight cases tested; preparation B2 was less active than preparation E1; the activity of preparation B3 is equal to that of preparation E1; the activity of preparation C1 is equal to that of preparation E1; preparation C2 was little stronger than preparation E1; preparations D1 and D2 caused nearly equal reactions; both were a little stronger than preparation E1.

No commercial preparation even approximated the strength of preparation E3 (0.01 mgm. of nitrogen to 1 cc.) and the activity of preparation A3 (the strongest of A, B, C, or D) was about equal to that of preparation E2 (0.005 mgm. of nitrogen to 1 cc.). As it has been our experience that even our most sensitive cases (with very few exceptions) require for therapeutic effect a maximum dose of pollen extract containing at least 0.025 to 0.05 mgm. of nitrogen while the less sensitive may need as high as 0.1 or 0.2 mgm. of active pollen nitrogen, it is difficult to see how a good result can be expected with the use of these comparatively weak commercial preparations except in the very sensitive cases, which constitute a relatively small percentage of the total. To obtain a full measure of relief stronger extracts should be used; however, if this is done, more caution must be exercised in their use as the more concentrated extracts are more apt to cause constitutional reactions (2).

The necessity for adequate dosage is well illustrated by the history of case 2273. This patient came to us in 1920 saying that he had been treated during 1919 with commercial preparation A for both early and late hay fever. The early hay fever was almost entirely relieved but the late hay fever was unaffected by the treatment. Unfortunately the first part of his history containing the record of his tests in 1920 has been lost. However, in 1921, he gave a positive eye reaction to an extract of timothy pollen (Cornell) containing 0.005 mgm. of nitrogen to 1 cc. while with ragweed pollen extract (Cornell) no eye reaction was produced by concentrations less then 0.1 mgm. of nitrogen in 1 cc. The ratio of his sensitiveness to the pollens, according to these tests was, thus, 20 to 1. In 1920 he received injections of commercial preparation A for his early hay fever and a Cornell extract of ragweed for his late hay fever.

The largest dose (in terms of nitrogen) of the latter preparation that he received was 0.16 mgm. Since our comparative tests indicate that the strongest solution of preparation A contained only about 0.005 mgm. of active nitrogen per cubic centimeter, it can be estimated that the patient received 30 times as much active ragweed pollen protein in 1920 as he had received in 1919. The results of the treatment were correspondingly different, for the injections of the Cornell extract of ragweed pollen produced the same degree of relief from the late hay fever as had been attained for the early hay fever with preparation A. During 1921 he received the Cornell extracts for both early and late hay fever, doing equally well with both; his maximum dose (in nitrogen) for the season was 0.04 mgm. of timothy pollen while his maximum dose of ragweed pollen was 0.16 mgm.

This case plainly illustrates that patients vary markedly in their degree of hypersensitiveness and also in the size of the dose necessary to relieve their symptoms.

Since this paper makes a plea for the use of larger therapeutic doses and more potent agents, it does not seem amiss again to caution the reader that this is attended with greater risk of producing constitutional reactions which in inexperienced hands can be dangerous (2).

<div align="center">REFERENCE</div>

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

III. ON CONSTITUTIONAL REACTIONS: THE DANGERS OF THE DIAGNOSTIC CUTANEOUS TEST AND THERAPEUTIC INJECTION OF ALLERGENS

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DEFINITION OF THE TERM

The term constitutional or general reaction is used to designate the group of symptoms occurring in allergic individuals after the absorption of an allergen and its transportation by the blood and lymph into the systemic circulation. Symptoms therefore occur in various organs and tissues affected by the allergen and may be protean in nature, since they depend upon the structures involved, which may differ with the individual and the allergen concerned. Such reactions may take place when the allergen is introduced through unnatural channels, as in the diagnostic skin test, subcutaneous or intravenous injection, or through natural channels, as after ingestion.

DATA USED

The data for the present paper are obtained more especially from a statistical study of 578 consecutive cases observed in 1920 and somewhat more generally from a personal experience over a period of ten years in the diagnosis and treatment of some four thousand allergic cases. The appended protocol gives the synopsis of the 61 cases with constitutional reactions occurring in 1920. Table 1 includes the facts relating to the 578 cases of that year.

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the human subject. It is the object of this paper to present and to correlate all the facts relating to such general reactions, as this type of work is being, and will continue to be, much more generally used and especially because of the dangerous and sometimes fatal results which may occur.

In all of these cases the diagnostic tests were made by the intradermal method and a definite diagnosis of allergy was based upon a marked cutaneous reaction: that is, a wheal with obvious pseudopod formation surrounded by a definite zone of hyperemia. It was further required that this marked positive reaction be repeatedly confirmed. The preparations used were those prepared by the writer standardized according to the nitrogen content. There are no data with which to compare the relative frequency of general reactions occurring with the hypodermic as opposed to the scratch method of cutaneous diagnostic test. It can only be pointed out that reactions do occur with the latter method, as, for example, the reaction produced by Rufus Cole with buckwheat, reported by Smith (1), using the scratch test. Some years ago the writer witnessed a constitutional reaction to timothy pollen where the pollen was applied to an abrasion on the forearm and dissolved in 0.8 per cent salt solution. Within five minutes there was a local reaction with a very difinite lymphangitis extending to the axilla, with intense itching in the cubital fossa and axilla. This was almost immediately followed by the general symptoms of erythema, urticaria, general pruritus, asthma and coryza, characteristic of the general allergic reaction. This lymphangitis is very characteristic of all marked positive reactions obtained on the anterior aspect of the forearm, even in the absence of general symptoms, and gives a clue as to the part the lymphatics generally must take in all local or general reactions.

It may be that constitutional reactions occur more frequently after the intradermal than after the scratch test. Since the general reaction depends solely upon the amount of the active allergen absorbed, it is possible that if such a difference does exist it will be found to be due to the use, in the intradermal test, of a more potent extract than is generally used in the scratch

ROBERT A. COOKE

PROTOCOL

CASE NUMBER	CLINICAL SYMPTOMS				INTRADERMAL TEST		
	Pollens giving		Other substances giving		Constitutional reactions from	Onset of symptom	Symptoms
	Asthma	Coryza	Asthma	Coryza			
2166			Orris	Orris			
2176		Timothy					
2177			Dust, cat				
2180			Horse	Horse			
2185		Ragweed	LePage	LePage	LePage	Immediate	Asthma, coryza, urticaria
2193		Timothy					
902		Ragweed		Orris, horse and dog epithelium			
2197		Timothy					
2199		Timothy					
2213		Timothy					
2388		Timothy					
2393		Timothy		Orris			
	Ragweed	Ragweed					
2415		Timothy					
	Ragweed	Ragweed					
2416		Ragweed					
2428	Ragweed	Ragweed					
2440		Ragweed					
2441		Ragweed					
2447			Horse				
2473	Ragweed	Ragweed		Orris, horse		•	
2479	Ragweed	Ragweed Timothy	Orris		LePage	Immediate	Urticaria
2492	Ragweed	Ragweed					
2520		Ragweed					
2526			Ipecac Linseed Buckwheat Feathers	Ipecac Linseed Buckwheat Feathers	Linseed	Immediate	Asthma, coryza, urticaria. Angioneurotic edema of neck lasting 2 days

PROTOCOL

Constitutional reactions from	Injection reacting	Onset of symptoms	Duration of symptoms	Symptoms	PROBABLE CAUSE	ASSOCIATED REACTION AT SITE OF INJECTION OR TEST
		ON SUBCUTANEOUS INJECTION				
Orris	4th	8 hours	2–3 days	Asthma	?	Marked
	5th	8 hours	10 hours	Asthma	?	Marked
Timothy	10th	1 hour	3 hours	Cough and urticaria	?	None
Cat epithelium	5th	8 hours	?	Asthma	?	?
Horse epithelium	1st	Immediate	6 hours	Asthma, coryza, urticaria, also nausea and headache lasted one week	Patient very sensitive	Marked
LePage	1st	Immediate	24 hours	Asthma, coryza, urticaria	Patient very sensitive	Marked
Timothy	8th	8 hours	12 hours	Pruritus of arms and neck	Change of extract	Marked
Ragweed	8th	12 hours	8 hours	Coryza, cervical glands	?	Slight
	9th	12 hours	8 hours	Coryza, cervical glands	?	Slight
Timothy	10th	Immediate	10 days	Asthma, coryza, cervical glands	Concentrated extract	?
Timothy	8th	Immediate	?	Urticaria	Concentrated extract	?
Timothy	5th	Immediate	4 hours	Coryza, erythema, headache and pruritus	Dose increased too rapidly	Moderate
Timothy	7th	1 hour	1 day	Asthma, coryza	?	Moderate
	10th	Immediate	1 hour	Asthma, coryza	?	?
Ragweed	17th	6 hours	2 days	?	Overdose	?
Ragweed	16th	?	1 day	Coryza	?	Marked
Ragweed	12th	24 hours	1 day	Coryza and edema of lip	Change of extract	None
Ragweed	14th	1 hour	?	Asthma, coryza, urticaria	Change of extract	?
Ragweed	16th	24 hours	6 days	Coryza, headache	?	?
Ragweed	18th	?	?	Coryza, edema of eyes	?	?
Horse epithelium	9th and 10th	?	?	Asthma	?	?
Horse epithelium and ragweed	10th	4 hours	2 days	Coryza	?	?
	11th	4 hours	2 days	Coryza	?	?
Ragweed					Patient very sensitive	Marked
Ragweed	10th	1 hour	3 hours	Asthma	Change of extract	Slight
	17th	½ hour	?	Asthma	Change of extract	?
Ragweed	7th	Immediate	?	Asthma	?	Moderate
	10th	Immediate	?	Asthma	?	?
					Patient very sensitive	Marked

ROBERT A. COOKE

PROTOCOL

CASE NUMBER	CLINICAL SYMPTOMS				INTRADERMAL TEST		
	Pollens giving		Other substances giving		Constitutional reactions from	Onset of symptoms	Symptoms
	Asthma	Coryza	Asthma	Coryza			
2274	Timothy	Timothy					
2276		Ragweed					
2283		Ragweed					
2284	Timothy	Timothy					
2285		Timothy Ragweed					
2286	Ragweed Daisy Dandelion	Ragweed Daisy Dandelion	Peach Celery Raspberry	Peach Celery Raspberry			
2290	Ragweed	Ragweed	Horse, cat Feathers, dust	Horse, cat Feathers			
2292		Ragweed		Orris			
2327		Ragweed					
2350	Ragweed	Ragweed					
2351		Ragweed					
2364		Ragweed	Horse				
2381	Ragweed	Ragweed	Horse	Horse			
2365	Timothy	Timothy					
2366		Timothy					
2367		Timothy					
2370		Ragweed					
2214	Ragweed	Ragweed	Feathers				
2216		Timothy					
2227		Timothy					
		Ragweed					
2233		Timothy					
2643		Timothy		Orris; dust			

PROTOCOL

	ON SUBCUTANEOUS INJECTION				PROBABLE CAUSE	ASSOCIATED REACTION AT SITE OF INJECTION OR TEST
onstitutional reactions from	Injection reacting	Onset of symptoms	Duration of symptoms	Symptoms		
Timothy	5th	Immediate	3 hours	Coryza	Change of extract	?
Ragweed	21st	?	?	Asthma, coryza, urticaria	Change of extract	?
Ragweed	7th	Immediate	?	Coryza	Directions not followed. Intervening doses omitted	?
Timothy	14th	Immediate	?	Asthma	Concentrated extract	?
Ragweed	18th	Immediate	?	Asthma, coryza, urticaria	Change of extract	?
Daisy, dandelion	2nd	Immediate	1 hour	Asthma, coryza	Patient very sensitive	Marked
Ragweed	8th	Immediate	2 hours	Urticaria in all	Patient very sensitive	?
	9th	Immediate	4 hours		Patient very sensitive	?
Ragweed	9th	Immediate	2-3 hours	Coryza	?	?
	13th	Immediate	2 days	Asthma	?	?
	15th	Immediate	?	Coryza, asthma	?	?
Ragweed	10th to	6 hours	36 hours	Urticaria	?	?
	20th	Immediate	?	Coryza	Change of extract	?
Ragweed	15th	Immediate	3 hours	Coryza, asthma, urticaria	Change of extract	?
Ragweed	19th	?	?	Asthma	?	None
Ragweed	16th	4 days	4 days	Urticaria	?	Moderate
Ragweed	18th	2 hours	?	Urticaria	?	Marked
Ragweed	12th	4 hours	8 hours	Asthma, coryza	Change of extract	Marked
	17th	3 hours	3 days	Urticaria, edema of eyes, coryza	Dose increased too rapidly	?
Timothy	11th	Immediate	12 hours	Asthma, coryza, urticaria	Dose increased too rapidly	?
Timothy	14th	8 hours	4 hours	Urticaria	?	?
	15th	8 hours	4 hours	Urticaria	?	?
Timothy	1st	Immediate	?	Coryza, erythema	Patient very sensitive	Marked
Ragweed	17th	6 hours	12 hours	Coryza, edema of eyes	Change of extract	Marked
Ragweed	6th	Immediate	4 hours	Asthma, coryza, urticaria	Concentrated extract	?
	20th	3 days	Several	Urticaria	Change of extract	?
Timothy	15th	Immediate	2 hours	Asthma, coryza, urticaria, edema of lips	?	?
	21st	1 hour	2 hours	Urticaria	Change of extract	?
Timothy	8th	Immediate	?	Asthma, coryza, urticaria	Concentrated extract	?
Ragweed	1st	Immediate	?	Severe abdominal pains	Patient very sensitive	Marked
Timothy	8th	½ hour	2 days	Asthma, coryza, urticaria, edema face	?	None
	13th	1 hour	8 hours	Asthma, coryza, urticaria, edema face	Change of extract	?
Orris	15th	24 hours	3 days	Asthma	?	Slight

PROTOCOL

CASE NUMBER	CLINICAL SYMPTOMS				INTRADERMAL TEST		
	Pollens giving		Other substances giving		Constitutional reactions from	Onset of symptons	Symptoms
	Asthma	Coryza	Asthma	Coryza			
2651		Timothy Ragweed	Horse, dog Buckwheat		Ragweed	Immediate	Coryza, asthma Headache, erythema
1058		Timothy			Timothy	Immediate	Coryza
2673			Egg, milk LePage, horse Rabbit, feathers		LePage	Immediate	General edema. Asthma, coryza, death from asphyxia
2681			Orris, wheat Dust		Orris	Immediate	Asthma, urticaria
2684			Dust, feathers				
2258	Ragweed	Ragweed					
2260		Timothy Ragweed					
2273	Timothy Ragweed	Timothy Ragweed					
2547	Ragweed	Ragweed					
2581		Timothy Ragweed					
2627		Ragweed	Orris		Ragweed	Immediate	Coryza, asthma, urticaria
2707			Feathers, dog, cat		Dog saliva	Immediate	Coryza, asthma, urticaria
2708		Timothy Ragweed	Chicken, rabbit Flaxseed, mustard		Flaxseed Mustard	Immediate Immediate	Asthma Asthma
896		Timothy		Cat			
2541	Ragweed	Ragweed					
2532	Ragweed	Ragweed					

test. This surmise is supported by the experiences of Brown (2) and of Larsen, Paddock, and Alexander (3), who found only 50 per cent of diagnostic efficiency using the dry preparation generally employed for the scratch test as compared with the fluid preparations used in the intradermal test.

HISTORICAL

A survey of medical literature discloses the fact that the year 1894, when diphtheria antitoxin was first introduced for general

PROTOCOL

	ON SUBCUTANEOUS INJECTION				PROBABLE CAUSE	ASSOCIATED REACTION AT SITE OF INJECTION OR TEST
Constitutional reactions from	Injection reacting	Onset of symptoms	Duration of symptoms	Symptoms		
Horse epithelium	4th	12 hours	2 days	Nausea	Concentrated extract	Marked
					?	Marked
					Concentrated extract	Marked
					Patient very sensitive	Marked
					Concentrated extract	Marked
Dust extract	5th	1 day	5 days	Urticaria	?	?
Ragweed	7th	Immediate	?	Asthma	?	None
	10th	Immediate	?	Asthma	?	Marked
Ragweed	10th	5 days	1 week	Urticaria	Dose increased too rapidly	?
Ragweed	16th	Immediate	?	Asthma, urticaria	?	?
Ragweed	2nd	Immediate	?	Coryza, asthma	?	Moderate
Ragweed	1st	1 hour	2 hours	Urticaria, edema of uvula	Patient very sensitive	?
					Concentrated extract	Marked
					?	?
					Concentrated extract	Marked
					Concentrated extract	Marked
	5th	Immediate	3 hours	Urticaria	?	?
Timothy	7th	Immediate	2 hours	Coryza, edema of eyes	Change of extract	?
	12th	Immediate	3 days	Coryza, edema of eyes, headache, urticaria	?	?
Ragweed	1st	Immediate	?	Coryza, erythema	Patient very sensitive	Marked
Ragweed	10th	24 hours	1 day	Erythema	?	?

use, marks the beginning of a period in which there are rather frequent reports of the violent reactions and sudden death following the injection of the antitoxic horse serum. The nature of the reaction was not understood. Gottstein (4) reported several such cases in 1896. In 1906, Rosenau and Anderson (5) state, "We have collected from the literature 19 cases of such unfortunate results and know personally of several more which have not been reported." Gillette (6) collected 30 cases Park (7), speaking of the frequency of such general reactions with diph-

theria antitoxin, says that since January 1, 1895, using either whole serum or the globulin fraction, thirty thousand cases had been injected, with collapse in two cases; that over a period of five years, when an immunizing dose was used in all scarlet fever cases, sixteen thousand cases were injected, with no collapse; and that in New York City, inspectors have given injections to one hundred and five thousand cases, with two deaths, but no record was kept of cases with reactions which were not fatal. Every death which did occur followed a primary injection.

The literature since 1913 has not been searched for the antitoxin reactions because the further collection of cases does not add materially to our knowledge of the reaction and because comparatively few of the reactions that have occurred have been reported. We are not interested here in the relative frequency of the allergic reaction to diphtheria antitoxin. Gillette (6) appears to have been the first to remark the similarity of the clinical reaction to the experimental anaphylactic reaction in the guinea-pig and he further noted the important fact that in man most of the severe reactions with dyspnea, edema, urticaria and pruritus, occurred immediately after the first injection, in this way differing from the experimental reaction.

Since this time the appreciation of the condition now known as human hypersensitiveness or allergy has greatly increased and, as we now look back over the literature, we find occasional instances reported of peculiar reactions to many different substances which chemically bear no relation to antitoxic serum. Indeed, a great many reactions, such as those of infants and children to milk, egg, and animal danders, were well known to the laity but occupied no place in medical literature. Peculiar drug reactions, known as idiosyncrasies, were well recognized, but they were not identified as allergies until 1916, when the writer noticed the relatively large number of such cases occurring among hypersensitive individuals and remarked the similarity of symptoms of drug reactions with those of foreign proteins in specifically hypersensitive persons. From our present vantage point we can easily appreciate the reactions obtained by Blakeley (8) with pollens of grasses, by Dunbar (9) with ragweed pollen and by Cole (1) with buckwheat.

Since the clinical conditions of bronchial asthma, hay fever, urticaria, angio-neurotic edema and the erythemas have been definitely recognized as manifestations of human hypersensitiveness and since it has been demonstrated that diagnoses can be made with the well known cutaneous reaction and that therapeutic effects can be obtained by injection, a great deal of work has been done and the results have been published, but a careful review of this literature since 1915 shows only a few records of the general or constitutional reactions following the use of allergens specifically applied to hypersensitive individuals for the purpose of diagnosis or treatment.   Rackemann (10) reported reactions in two cases following therapeutic injection so fextract of horse dander.   Walker (11) mentions but incidentally a general reaction following a therapeutic injection of an extract of flaxseed.   Gustenberger and Davis (12) reported a reaction with egg protein, the intradermal method being used for testing. The writer (13) has called attention to the dangers of the constitutional reactions on injection and has noted their frequency with pollen extracts as being 3.75 per cent in 4192 injections in 339 hay fever cases up to January, 1916.   The occurrence of many other such reactions is personally known to the writer but they have not been reported in the literature.

SYMPTOMS OF THE CONSTITUTIONAL REACTION

The symptoms of general reactions in allergic individuals are entirely distinct and apart from the symptoms that occur in the normal man, even though the latter be given one hundred thousand times the amount of allergen (for example, horse serum). Allergic symptoms are as characteristic as those of a typical lobar pneumonia.   The onset of constitutional symptoms may be immediate or they may be delayed up to five days.   Discussion of this point is made in greater detail in the section on "Varieties of the Reaction."   In general, the symptoms are those of the various clinically recognized allergies and in any individual case they are usually those from which the patient suffers, plus certain manifestations in tissues not reached by the allergen under ordinary exposure.   Thus case 2185 has asthma,

coryza and urticaria from eating fish, and asthma and coryza from handling fish glue (LePage glue). He showed identical symptoms in the constitutional reactions after a test and after a therapeutic injection of the sterile solution of LePage glue. On the other hand case 2197, who has clinically only hay fever from timothy pollen and never asthma, developed coryza and in addition asthma and glandular swellings in the neck, following the subcutaneous injection of timothy pollen extract. Case 2180 had, in addition to the usual clinical symptoms, nausea and severe headache for a week following the first injection of horse dander extract. We can group constitutional symptoms as those that are usual and those that are infrequent.

Usual symptoms

Coryza. This term is meant to include the ocular symptoms of corneal injection, lachrymation and itching, as well as the nasal symptoms of discharge, sneezing, and edema of the mucous membrane causing obstruction. The term "Allergic Coryza" has been employed by the writer (14) to designate all forms of vasomotor rhinitis that are allergic in nature. Coryza is a particularly common symptom of general reactions and it may occur on the ingestion of allergens as foods and drugs, as well as when these substances are injected for test or treatment. It is more common, however, with substances such as pollens. that are naturally absorbed by inhalation.

Asthma. This is somewhat more common in cases with clinical asthma but it does occur in clinically non-asthmatic cases, as no. 2197 mentioned above. Asthma is a symptom of the bronchial edema which makes the general reaction dangerous and which may be the cause of death from asphyxia.

Urticaria. This is a very usual manifestation as would be indicated by the frequent occurrence of the immediate skin reaction on test. It is prevented from being a usual clinical symptom by the fact that a sufficient amount of allergen is not absorbed through the respiratory mucous membrane and carried by the systemic circulation to the skin.

Erythema. In some cases the skin becomes scarlet without the appearance of urticarial wheals.

Pruritus. This condition usually accompanies the urticaria or erythema but does exist without either. One case not included in the 1920 series regularly (at least six times) had a very marked pruritus ani within fifteen minutes after an injection of timothy pollen extract.

Edema. The angio-neurotic type of edema may occur in any part of the body, and when once developed, it takes two to four days for the condition to subside. In cases of allergic coryza it is most commonly observed in the tissues of the upper and lower lids so that the eyes may be entirely closed. Occasionally the sclera is also involved and the edematous scleral tissue may protrude between the lids. Edema of the glottis has not been identified though it may have occurred in the fatal case cited later in this paper, and edema of the gastro-intestinal tract may explain some of the cases in which abdominal pain is a symptom.

Cough. This may occur independently of asthma though it is often associated with it. It is violent and paroxysmal like the cough of pertussis and seems to be due to laryngeal irritation.

Infrequent symptoms

Glandular enlargement. This is noted twice in the cases included in the protocol, nos. 902 and 2197. It has not been noted by me in more than six cases. While it is noted as part of an immediate reaction in case 2197, it is certainly not itself a noticeable phenomenon in less than three hours and the swellings usually last three days. The submaxillary and cervical glands, especially the anterior chain, are the only ones that have been observed, except in one case not included in the protocol, in which, following one of the injections, the preauricular glands were so enlarged that the condition was at first diagnosed as a parotitis. In this case following the next injection the same glands became swollen and tender and with them the cervical group. When the same reaction occurred a third time after injection, the time interval in all three instances being about twenty-four hours,

there could be no mistaking the relation between injection and glandular enlargement.

Headache. This may be of a mild type, frontal or occipital, and, like the glandular enlargements, does not develop immediately. It may persist, as in one case, no. 2180, for a week. On the other hand, headache may be excruciatingly severe and of a migrainous type. The writer experienced one such reaction in himself following an injection of horse dander extract. The headache developed in ten hours and gave the sensation truly described as "splitting." It lasted six hours. The writer is otherwise not subject to such headache.

Fever. This, together with chilliness, has been complained of in a few cases but it has not been verified by actual readings except in the case with preauricular adenitis, when a temperature of 101°F. was observed with each of the three glandular reactions. In this case it began in six hours and lasted for twenty-four hours.

Nausea. Nausea sometimes accompanied by syncope and vomiting is not very usual. With violent immediate reactions of the usual type, vomiting may occur inside of an hour and under these conditions it is so copious that there must be an extraordinarily large secretion from the gastric mucosa to account for the volume. In one case, not included in the protocol, in which a constitutional reaction with asthma took place after aspirin by ingestion, the vomiting followed in twelve hours and with it the attack ceased. This was also the usual clinical course, according to the patient, whose attacks had for some time followed the use of aspirin taken for headache on the advice of his physician.

Diarrhea. No cases in the 1920 series exhibited this symptom. I can recall two cases in which diarrhea followed the injection of ragweed pollen extract. The attacks started in six hours and lasted from twelve to twenty-four hours. The regularity of recurrence after injection in both cases is the sole reason for considering it a general reaction. There were no other constitutional symptoms in these two cases.

Acute abdominal pain. This symptom has been noted in a few cases not in this series and has been attributed to an angioneurotic edema of the gastrointestinal tract. Acute abdominal pain, cramplike in nature, over the lower abdomen, developed within one hour after the first ragweed injection in case 2227. The case was not observed during this attack and there is no record of its duration. It was not associated with vomiting or diarrhea. This may have been a type of reaction similar to those discussed under dysmenorrhea. The menses were absent in this case on account of the fact that this patient, a girl, was barely thirteen years of age and the menses had not yet been established.

Dysmenorrhea, or rather an untimely and scanty menstrual flow, following acute cramplike pains in the lower abdomen is recalled in the case of two women aged twenty-six and thirty-eight, respectively. In both of these cases the symptom was part of an immediate reaction with asthma, coryza and urticaria, the menstrual flow itself not being apparent until three hours later and lasting only one day. Among many pregnant women tested and treated no such symptom has ever appeared, but extreme caution is always taken in such cases, for abortion might be induced.

Syncope. This was not noted in any of the 1920 series. It does occur as an immediate effect, namely within one hour, and is usually associated with nausea and vomiting. I recall but one case in which it occurred alone. Reference is not made here to those occasional cases of syncope due to mental or nervous instability in which the patient will give a history of similar attacks following the sight of a hypodermic needle or a drop of blood.

Cardiac collapse. I recall but one case in my entire experience in which this condition took place as a primary symptom. Of course cardiac dilatation and vasomotor collapse do occur secondarily in the fulminating types of reactions with extreme dyspnea and partial asphyxia. But in the case referred to above, there developed, within one-half hour and with an absence of all usual general symptoms, a profound weakness and prostration, pallor and sweating without the loss of consciousness of syncope.

The heart rate was 140 and the pulse was imperceptible, with cold skin and marked sweating.   One milligram of strophanthin was given intramuscularly with adrenalin chloride 15 minums. Except for the adrenalin effect the condition was normal within one hour.

From such a categorical list of the symptoms of a general reaction, one draws but a hazy idea of the pictures of the actual reaction in the individual case, but, as case histories will be cited later illustrating the various points to be brought out, no attempt will be made here to fill this deficiency.

VARIETIES OF THE CONSTITUTIONAL REACTION

The constitutional reaction can conveniently be considered as occurring in two forms, the immediate and the delayed.

1. The immediate general reaction

The immediate reaction occurs within one hour after the allergen is introduced.   This more or less arbitrary time limit has been adopted by the writer because in practical clinical work one sees a very large group whose reactions fall well within this limit of time, in fact well within one-half hour.   In some cases it ensues on the instant (within one minute) and the severity of the symptom is in direct proportion to the brevity in time of onset. The sooner the symptoms begin, the greater their intensity and the greater the danger of a fatal result.   On the other hand if cases do not react within the hour, the reactions are usually delayed for a period of at least six hours, and may not make their appearance for five days.   No definite reactions have been identified after a longer interval though there seems to be no reason why they should not so occur.   The protocol of the constitutional reactions accompanying this paper shows only 3 cases, nos. 2364, 2381, 2473, with 4 reactions, in which the reaction appeared after one hour and under six hours, out of a total of twenty cases with 44 delayed reactions; i.e., after six hours.   While this differentiation of immediate and delayed reactions is, for the present at least, based upon confessedly arbitrary grounds, it

serves a definite clinical purpose in that it separates the intense and dangerous reactions from those that are merely subjectively disagreeable.

Allergens may cause immediate constitutional reactions by whatever path they may be introduced systemically; that is, after test, after injection or on ingestion. Since these immediate general reactions may occur when the cutaneous reaction is negative as well as when positive, it will be well to discuss separately the general reactions occurring with allergens which give a positive skin test and those occurring with allergens which give a negative skin test.

a. *When the cutaneous test is immediately positive.* The immediately and genuinely positive intradermal test is pathognomonic of a cutaneous hypersensitiveness and indicates an accompanying clinical hypersensitiveness of the mucous membranes of the respiratory tract in approximately 95 per cent of the cases reacting by test to pollens and animal epithelia. In other words, the intradermal test reactions agree with the clinical histories or can be clinically substantiated in 95 per cent of the cases reacting to the pollens and animal epithelia. This has been well shown by Vander Veer in the following table based on studies of cases in our clinic.

	POSITIVE REACTION WITH POSITIVE HISTORY	NEGATIVE REACTION WITH NEGATIVE HISTORY	NEGATIVE REACTION WITH POSITIVE HISTORY	POSITIVE REACTION WITH NEGATIVE HISTORY	REACTION AND HISTORY CORRESPONDING
					per cent
Pollens	155	29	4	4	96
Animal epithelium	20	17	2	0	95

When 0.01 cc. of extract is injected intradermally for the test according to the usual technic, a general reaction may follow within a few minutes. Ten such cases are cited in the protocol. One had two reactions with different allergens. Thirty-one cases in the protocol gave 42 immediate general reactions on therapeutic injection of the allergen. This is due directly to an overdose and the time of onset and symptoms are exactly similar to reactions from the test. Following is the history of the case in which death resulted from the test.

Case 2673.   Boy, three years of age, developed an attack of asthma at the age of eighteen months.   The attack started with cough, then dyspnea and vomiting followed.   There was no coryza.   The dyspnea became increasingly severe until he passed into collapse and a semi-conscious state with a pulse rate of 160 and marked cyanosis.   From this time on he was constantly asthmatic with exacerbations.   These severe attacks lasted from one to seven days and occurred about once a month.   As he would vomit immediately after ingestion of egg, no eggs had been used for over a year.   He had mild urticaria at the age of one year.   Eczema had been present for the first two years of life.

Physical examination.   The patient was an undersized, poorly-nourished boy.   Respiration was labored.   He appeared anemic.   There was nothing else of note except the sibilant sounds and râles throughout both lungs and a marked double Harrison's groove.

On the first visit he was tested with milk, egg and cereal preparations, eight tests in all.   Tests with very dilute egg protein were only suggestive but the casein preparation gave a marked reaction.   The cereal preparations were negative.   Two days later he was tested with more concentrated egg preparations.   Ovomucoid gave a moderate reaction and egg white globulin a marked reaction.   The meat extracts were negative.   The next day he was tested as follows: (The decimals indicate milligrams of nitrogen per cubic centimeter of solution; 0.01 cc. was used to test.)

Orris root ....0.1 negative	Chicken epithelium.. 0.5 marked
Dust extract .... negative	Horse epithelium.... 0.04 slight
Dog epithelium......0.1 negative	Horse serum......... 0.1 marked
Cat epithelium .....0.1 negative	Rabbit epithelium... 0.2 marked

No untoward results had followed these tests.   The reactions all subsided within twelve hours.   Two days later, November 29, 1920, he was better than usual.   The following tests were made:

Tests

Ragweed....................................0.1	Vanilla
Timothy.....................................0.1	Chocolate
Horse epithelium............................0.4	Peanut
LePage glue.................................0.1 marked	Cocoanut

Within two minutes it was noticed that the reaction at the site of the LePage glue test had spread up and down the arm and there were many

fine urticarial spots appearing all over the arm.   The boy then suddenly broke out in a general rash, his face began to bloat with an edema, until his eyes were closed.   Cough and dyspnea were marked for a minute, he was deeply cyanotic and respiration ceased, though respiratory efforts continued for a minute longer.   Artificial respiration was attempted but no air could be made to enter or leave the chest.   The heart continued to beat for a minute after respiration ceased.   He had been given 1.0 cc. of adrenalin (1:1000) intravenously at the onset of the attack and strophanthin 0.125 mgm.

In my opinion this was a genuine allergic death from asphyxia and not due to any associated condition of status lymphaticus. I have attributed this death to the LePage (fish glue) solution because the patient was not very sensitive to the horse epithelium as shown by the test on a previous day and because the marked local reaction and urticaria started about the site of the LePage test.   In this case the LePage solution used contained 0.1 mgm. of nitrogen per cubic centimeter; 0.01 cc., at most it can be supposed 0.02 cc., was injected.   This means that death in this case was caused by a dose containing only 0.001 to 0.002 mgm. of nitrogen.

On ingestion of the allergen in its natural form, where the cutaneous reaction has been positive, a reaction may occur immediately and, if the buccal, esophageal and gastric mucous membranes react as well, either the substance cannot be swallowed at all or it is very quickly vomited.   This is the condition in many of the egg allergies of children.   Such mucous membrane reactions are in reality local manifestations but in certain of these cases absorption of the allergen into the systemic circulation may take place through the hypersensitive buccal or lingual mucous membrane.   In a child of eight years, exquisitely hypersensitive to egg, a mild urticaria developed fifteen minutes after a piece of cake containing egg had been placed against the tongue for a minute.   The saliva was not swallowed and the tongue remained protruded until she rinsed her mouth out thoroughly with water at the end of the experiment.   The tongue itched and was very red and slightly swollen.

There are cases in which the gastro-intestinal mucous membrane appears not to be sensitive to a substance which may produce a general reaction when the substance is eaten. An example of such an occurrence is presented in the history of case 2826. This individual, a woman of thirty-three years of age, exhibited the symptoms of edema of the lips and face within a few minutes after the ingestion of two hazel nuts and after twenty minutes she experienced a severe attack of asthma that lasted for two hours. She had had such a clinical reaction on two other separate occasions. Urticaria and pruritus had never been a part of this clinical reaction. There were no gastro-intestinal symptoms either at the time or subsequently. Other nuts besides hazel nuts could be eaten with impunity.

b. *When the cutaneous test is negative.* Occasionally, even when the skin test is negative, a general immediate reaction ensues after cutaneous test, after subcutaneous injection, or after ingestion, that is unmistakable as to its cause, as will be shown in the case 1766 to be cited. This can be explained on the assumption of a complete absence of skin allergy with a hypersensitiveness limited to the respiratory mucous membrane. In my experience this has occurred only with the drugs and particularly with aspirin. The clinical history bears out the above assumption, for in the cases of aspirin allergy, if urticaria has been one of the clinical symptoms a positive cutaneous reaction is obtained but otherwise the cutaneous reaction is negative. The only difference between the general reaction that occurs after ingestion of aspirin and that following its injection is that twenty to thirty minutes elapse before the onset of symptoms when the drug is ingested and only two to five minutes when it is given by injection. This seems to be merely a matter of rapidity of absorption. In all the aspirin allergies observed, with or without positive skin test, there has been no immediate reaction of the alimentary mucous membrane when the drug is ingested but the symptoms of coryza and asthma are the same as when the drug is injected. Vomiting has occurred in several cases eight to twelve hours after ingestion and this act usually terminates the attack. This indicates a central effect. The following case

illustrates the general reaction occurring on test, on injection and ingestion of the drug where the cutaneous test itself was negative.

Case 1766. Age forty, female. This individual has had three attacks of asthma in the last three months but each one has been extremely severe. The first attack started at 3 p.m. She had been in usual health and was on the street at the time and remembers being just able to crawl to her room. She described the sensation of strangling as though a rope were being pulled tight about her neck. She evidently became unconscious for she found herself on the floor at 1:00 a.m. She did not recover for two weeks during which time her chest was sore, as though she had been beaten. There was cough and nausea for several days, no headache, no fever and *no urticaria*. In the next attack eight weeks later she was seen by a physician within one-half hour after the onset of the attack. Adrenalin was given and she was taken to a hospital. The symptoms were the same as in the first attack. The attack was controlled and lasted altogether three-quarters of an hour but she was ill for two days with weakness and prostration. The third attack, exactly similar, occurred four weeks later. It started at 10 p.m., and had lasted for three-quarters of an hour when morphine was given. This attack incapacitated her for three days. Each of these attacks had occurred on the third or fourth day of the menstrual period. Physical examination was practically negative. Closer questioning then revealed the fact that she had been given a prescription for menstrual headaches and that she had taken one of these capsules twenty to thirty minutes before each of the three attacks of asthma. They contained strychnin, quinin and aspirin. Quinin bisulphate and aspirin in solution were injected intradermally. The amount introduced was less than $\frac{1}{1000}$ grain of each. At the site of test there was absolutely no reaction, either immediate or delayed. Within fifteen minutes after the test the patient began to cough violently, complained of a sensation of filling of the throat and thickness of the tongue, numbness of the hands with itching and dryness of the lips and throat. There was no erythema or urticaria. These symptoms disappeared in one-half hour. Six days later a test was again done with $\frac{1}{10000}$ grain of aspirin. There was no reaction at the site of test. A similar but milder constitutional reaction followed immediately. Five days later quinin was tested with negative local and constitutional reaction. Then $\frac{1}{10000}$ grain of aspirin was given by subcutaneous injection and the same immediate general reaction ensued in five minutes, with no reaction at the site of injection at the time or later.

2. Delayed constitutional reactions

Under "Immediate Reactions" the reasons were given for the establishment of the time limit of one hour for such reactions. Reactions occurring after this time are considered as delayed.

a. When the cutaneous test is immediately positive. The writer has observed a number of instances in which constitutional symptoms have occurred after an incubation period of one hour or more following a subcutaneous injection of the allergen. In no case was this "delayed" general reaction observed to follow the preliminary test or the first subcutaneous injection but always after a number of injections (usually 8 or 10) had been given.

In the protocol appended to this report there are 20 cases in which 44 delayed general reactions occurred. It will be seen that these delayed reactions were always observed after repeated previous injection had been made.

In striking contrast with these observations are the 10 cases in which constitutional reaction followed the preliminary test. In all of these the general reaction occurred immediately.

It must not be inferred from these statements that an immediate general reaction cannot follow the later injections. Indeed such an occurrence has been frequently observed. The length of the time interval between the injection and the onset of symptoms in the treated cases seems to depend entirely upon the amount of allergen injected, for a sufficiently large amount of the allergen may at any time produce an immediate reaction. Rackemann's (10) reported cases bear out this observation. In my own series, case 2292 of the protocol had given delayed general reactions as shown by urticaria, thirty-six hours after each injection from the tenth to the twentieth. But with the twentieth dose (change to new extract) an immediate coryza developed. Another interesting phenomenon in connection with delayed constitutional reactions in which the delay is induced by therapeutic subcutaneous injections is that a local reaction at the site of injection may be entirely absent if injections have been given in approximately the same site, but here again a sufficiently large or a sufficiently concentrated injection of the

allergen will suffice to produce an immediate reaction at the injection site, as well as an immediate constitutional reaction. The phenomenon of the delayed general reaction appears to be an expression of the purely relative insensitiveness which the writer in another article (15) in this Journal has chosen to designate by the term "Hyposensitization." In such cases a marked positive immediate cutaneous reaction is always obtainable with sufficiently concentrated extracts. This induced delay of the constitutional reaction in hyposensitive cases may obtain whether the allergen is absorbed from a subcutaneous injection or from an intracutaneous test. It likewise holds when the allergen is absorbed by ingestion as shown by the following history.

Case 1107. Age two years when seen in 1917. The first time egg was ever given, about one year previously, his face, lips and tongue became swollen, almost at once. He choked, coughed and became dyspneic and cyanotic, vomited several times and complained of abdominal pains. The father has early hay fever and the mother attacks of urticaria. On skin test the boy reacted markedly to the proteins of egg white but not to egg yolk. He was given injections of egg proteins. After seven injections he could eat egg in pudding in small amounts without trouble. After ten injections he could take five teaspoonfuls of soft boiled egg without trouble. Four more injections were given in this year and he could then eat one egg every other day without trouble until April, 1919, when he developed cough and hoarseness. Eight more injections were given. After this it was noted that he could eat an egg every other day without symptoms. When he was fed one egg every day he developed a harsh hollow cough by the second day, thus showing the delayed constitutional reaction. When he was put on two eggs a day he developed edema of the face about eight hours later—again a delayed constitutional reaction. All symptoms of cough and edema disappeared forty-eight hours after egg was discontinued.

b. When the cutaneous test is negative. Delayed constitutional reactions occurring to allergens to which cutaneous reactions are negative belong to an entirely different group from the hypersensitive cases under consideration. The discussion of them is therefore reserved for a later paper.

CAUSES OF CONSTITUTIONAL REACTIONS

1. Mode of introduction of the allergen

All the clinical studies in allergy here presented have been carried out by means of the intradermal test and the subcutaneous injection. Resort is never had to intravenous injection. The rate of absorption from the skin and subcutaneous tissue is approximately the same. General reactions from intravenous injection would undoubtedly be more immediate and more severe. In making the cutaneous and subcutaneous injection it is perfectly possible that at times the point of the needle should lie in a small vein or lymphatic vessel and undoubtedly this does occur at times and may account for some of the constitutional reactions resulting, but such an occurrence is rare and seems unavoidable and is of no practical account in comparison to the causes to be discussed under 2, 3, and 4.

2. The reactivity of the individual

There are all grades of reactivity varying from those that give very marked positive reactions with the weakest dilutions to those that barely can be said to be positive with the most concentrated extracts. Naturally those reacting violently to the weakest dilutions—with the pollen extracts a concentration of 0.0005 mgm. of nitrogen per cubic centimeter—are most susceptible to the constitutional reactions even when tests and injections are properly carried out. An appreciation of this variation in reactivity is of the utmost practical importance in the therapeutic management of the cases.

3. Activity of the allergens

Great difficulty was experienced in the past by the instability of the extracts, notably the pollen extracts. As will be observed by a glance at the protocol, many of the constitutional reactions followed a change of extract; that is, from one that had been in use a few weeks to a new and freshly prepared one. Even by repeating the tests and by diminishing the dose accordingly it

was not possible always to take sufficient account of the deterioration that had resulted and general reactions ensued. In 1921, with the use of extracts prepared by Coca (16) as given in another article in this issue this great disadvantage has been overcome and we now have preparations that have remained practically stable for over ten months.

4. Concentration and dosage of the extracts

The concentration of the allergen used in the test and the total amount given by injection are of paramount importance. Discussion of these questions is reserved for a later paper.

5. Cumulative effects

a. With the same allergen.

Case 2286, cited in the protocol, reacts clinically and by test to a number of curious and unusual allergens, such as celery, skin of peaches, raspberry and in addition has hay fever and asthma from the pollens of daisy, dandelion and ragweed. On May 6, 1920, she was tested intradermally with extracts of daisy, dandelion, ragweed, celery, timothy, apple, lilac, wistaria. The first four extracts gave marked cutaneous reactions. She was then given by injection daisy and dandelion, 0.001 mgm. of nitrogen of each. There was considerable local reaction for twenty-four hours. No constitutional reaction developed. Two days later the same dose of the same extract of daisy and dandelion out of the same bottle produced a constitutional reaction within one hour with asthma, coryza and urticaria as the symptoms. It is difficult to interpret this reaction on the second injection except on the assumption of a cumulative effect, for on the first visit the patient had actually received more than on the second as the tests were done only a short while before the first injection was given.

b. With different allergens. If a hypersensitive patient is tested with ten to sixteen different extracts and happens to be acutely reactive to four or six of them it is the writer's impression that a general reaction is much more likely to ensue as a result of the sum total effect of all the allergens exerting their influence upon the same reacting mechanism. I have no case that affords

absolute proof of this assumption. The suggestion is offered because it has a definitely practical bearing and will serve as a warning against the carrying out of too many tests at the same time.

6. Allergens which have caused constitutional reactions

A list is here given of all the allergens that, in the writer's experience have definitely caused constitutional reactions, either on test, injection, or ingestion:

LePage	Dog epithelium	Orris
Ovo-mucoid	Dog saliva	Dust extract
Egg albumin and globulin	Flaxseed	Aspirin
Pollens, all kinds	Linseed	Quinine
Horse epithelium	Cottonseed	Ipecac
Horse serum	Mustard	Chicken epithelium (feathers)
Rabbit epithelium	Pepper	Duck epithelium (feathers)
Rabbit serum	Buckwheat	Goose " "
Cat epithelium	Wheat	Nuts (peanut, hazelnut)

Having thus outlined the causes of the general reaction it is obvious that they may be avoided by proper care. Granted than an occasional injection or a test may be accidentally delivered in a venule a serious reaction will not occur if; first, extracts used are sufficiently dilute; second, injections are not repeated too frequently; third, tests, not to exceed six or eight, are made at one time. It is not the purpose of this paper to outline instructions for the use of extracts made according to the directions of Coca in another paper in this issue. The object here is to call attention to the dangers inherent in their use. Specific directions for their use will be given in a subsequent paper.

FREQUENCY OF CONSTITUTIONAL REACTIONS

It may be said that in the series presented general reactions have ensued with great frequency and this is true for the year 1920. But it may be added in extenuation, that these results occurred in a serious attempt to determine the limits of diag-

nostic reactivity and to define the limits of therapeutic dosage. Furthermore, many hitherto unknown allergens have been discovered and in the positive identification of these reactions have occurred.   The justification, if such is needed, lies in the fact that the study and correlation of the facts has enhanced our knowledge of the nature of allergy, as well as of the technic of diagnosis and treatment.   The following table is based upon our series of 473 consecutive allergic cases studied in 1920.   All of the cases giving constitutional reactions are included in the appended protocol.

TREATMENT OF CONSTITUTIONAL REACTIONS

A thorough knowledge of the treatment of the reactions should be had by every one attempting this type of study.   First of all, one should quickly recognize the onset of the symptoms usually shown by the extensive urticarial wheals developing about the site of the injection or test or by a beginning erythema or short paroxysmal cough or increasing dyspnea.   At once a tourniquet should be tightly applied about the arm above the site of the tests or injections in order to prevent the transportation of more allergen to hypersensitive tissue through the systemic circulation by means of the lymph or blood stream.   Adrenalin 1:1000, 1 cc. in adults, in children 0.4 to 0.6 cc., should be given at once subcutaneously or, if the reaction is severe, an intravenous injection of the same amount.   If there is a continued increase of symptoms the dose should be repeated in two to five minutes. The writer has never seen any ill effects from these large doses of adrenalin and is confident that if it is used often enough and in large enough dose, the serious results of a reaction can be avoided. Adrenalin is the very best drug available.   In the presence of a cardiac dilatation from violent respiratory effort or in the presence of vasomotor collapse, strophanthin, 1 mgm., intravenously, (in children, a proportionate dose) should be given without delay.   When the attack is controlled and has passed its peak of severity, morphin in proper doses may be used.   The writer has never seen any advantage in the use of atropine in these attacks and the use of this drug militates against the best effect of strophanthin, should it be required.

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

IV. NEW ETIOLOGIC FACTORS IN BRONCHIAL ASTHMA

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The cutaneous test is accepted today as a diagnostic procedure in the study of human hypersensitiveness. The marked immediate cutaneous reaction that can be confirmed repeatedly is the evidence of the hypersensitiveness of the skin. In the writer's article "On Constitutional Reactions," in this issue of the Journal (page 000), it is shown by a table of cases that the typical cutaneous reaction is likewise indicative of clinical hypersensitiveness in at least 95 per cent of the cases reacting to the extracts of such airborne substances as pollens, powdered root of orris and animal danders.

Bronchial asthma was early recognized as the chief clinical manifestation of the hypersensitive state in human beings and many of the cases were readily diagnosed by testing with extracts of such substances as those mentioned above, easily conceived and long known clinically to be important excitants of an attack.

For those cases not thus easily diagnosed an explanation was sought along two lines, first that it was due to bacterial proteins acting as allergens and secondly that the paroxysm of asthma was a reflex effect.

1. BACTERIAL PROTEINS AS ALLERGENS

The thesis has been advanced that the asthmatic paroxysm can be induced by the absorption of bacterial proteins which, acting as allergens, produce the symptoms just as any other

foreign proteins in the specifically hypersensitive man. This
was perhaps a natural corollary to the work already done on
foreign proteins in hypersensitive humans by Noon, Schloss,
Cooke, Goodale, Longcope, Talbot and others.

Since Walker is largely responsible for the term "Bacterial
Sensitization" or "Bacterial Asthma," meaning thereby that the
bacterial protein, acting as an allergen, is a basic etiologic factor
in one of the clinical allergies as bronchial asthma, let me review
his work for the facts by which he concludes the thesis has been
sustained. He began with a study (1) of the bacterial flora of
the nasal and bronchial secretions of asthmatics and recovered
principally *Staphylococcus albus* and *aureus*, *Micrococcus tetra-
genous* and *catarrhalis* and a new diphtheroid organism. He
carried out agglutination tests with *Staphylococcus aureus*, using
the serum of 80 asthmatics. In only 3 cases was the test carried
out with the serum of the individual from whom the organism
was obtained. In 2 of these cases there was agglutination and
in 1 there was not. Out of the 80 sera tested, 54 agglutinated,
some in dilutions of 1:50 or less and some 1:100 or higher up
to 250. No mention is made of any control with the serum of
non-asthmatic individuals. He concludes (page 379) that no
inferences can be drawn as to the relations between the isolation
of *Staphylococcus pyogenes aureus* from the sputum and nasal
secretions of patients and the agglutination tests of the sera of
those patients.

In his Study III (2) on the sensitization of patients with
bronchial asthma to the bacterial proteins, as demonstrated by
the cutaneous reactions, he reports on 100 asthmatics. He used
a bacterial powder obtained by centrifuging the bacteria from
normal saline in which they were washed. They were then
washed twice in alcohol, afterward in ether and then pulverized.
The powder was put on a scratch and dissolved in $\frac{N}{10}$ sodium
hydrate. He observed five types of reaction, three of which he
calls positive. One of these positive types is the urticarial
wheal which we recognize as characteristic of the positive allergic
cutaneous reaction, the second a small papule with surrounding
erythema, the other consists solely of hyperemia. These reac-

tions develop in one-half hour at which time the reading is made. There is no reason given for considering them positive except that 67 cases in the group were negative, that is did not give the same hyperemia. Ninety-seven cases were tested with *Staphylococcus aureus* and 19 were considered to be positive. Nine of the 19 gave an urticarial wheal as the evidence of a reaction. There were no controls on non-asthmatic cases reported. His conclusion is that "In patients with bronchial asthma, positive reactions with the protein of *Staphylococcus aureus* are more common than with the protein from other bacteria."

In the next study (3) dealing with the subject Walker turns to "complement fixation and precipitin reactions with the serum of bronchial asthmatics who are sensitive to the proteins of wheat . . . . and bacteria, using these proteins as antigens, and the cutaneous reaction as an indication of sensitization." Six cases of asthma are presented which gave a cutaneous reaction to *Staphylococcus aureus* that was called positive. Three of them showed a positive complement fixation and 3 were negative; but 2 of the 3 negative cases gave positive precipitin tests, while the third case, designated as M. S. was negative. Speaking of this case, Walker says: "Since M. S. was relieved of asthma by *Staphylococcus pyogenes aureus* vaccine, and the case, J. H. N., was greatly improved during treatment with desensitizing doses of *S. pyogenes aureus* protein, the asthma in these 2 cases would seem to be caused by staphylococcus pyogenes aureus." The organism is not reported as having been sought in the nasal or bronchial secretions of this case M. S. In conclusion, Walker states (page 265) that it is not possible to correlate the results obtained with complement fixation, specific precipitation and cutaneous tests. But he then asserts in Study XIII (4) that "the cutaneous reaction has proven to be of great value in determining the cause of asthma from bacterial protein as from other proteins." He then proceeds to a discussion of "the relationship between cutaneous reactions, serum agglutination tests, and bacterial examination of the sputum and nasal secretions in determining the part *Staphylococcus pyogenes*

aureus and *albus* may play in the cause of bronchial asthma."
The author states that 30 cases form the chief basis of this
paper, whereas only 21 appear accounted for in the text, 5 under
protocol I, 2 under protocol II, 5 under protocol III, 3 under
protocol IV, used as controls, and 6 other cases without pro-
tocols. In conclusion he says: "The cutaneous test has proven
to be the safest and best test for determining the bacterial cause
of bronchial asthma."

Let us examine these cases and see what constitutes the proof.
We must eliminate the 3 control cases, leaving 18 for consider-
ation, 13 of which gave a positive cutaneous reaction to *Staphylo-
coccus aureus*. In only 4 of the 13 cases was the organism
recovered. Nine of the 13 were treated with *Staphylococcus
aureus* vaccine and the asthma relieved at least temporarily,
but in only 3 of these 9 was the organism recovered. In contrast
to this there are 5 cases with a negative cutaneous reaction in
3 of which the organism was recovered and all did just as well
under treatment with *aureus* vaccine. On the other hand, 12
of these 18 cases gave a positive agglutination test and the organ-
ism was recovered in 7 of the 12. Eleven of the 12 were treated
with aureus vaccine and relief of asthma was obtained in 10 of
the 11 cases. In other words, in would be very difficult for any
one to attempt on the basis of such figures to maintain that the
cutaneous reaction was any more efficient than the agglutination
test. From this point on all of Walker's papers are based on
his belief that he has proven the cutaneous reaction in bronchial
asthmatics to be as etiologically diagnostic with bacterial pro-
teins as with other proteins, such as egg, wheat, pollens and
animal danders.

This critical review of Walker's studies can lead only to the
conclusion that he has not brought forth any proof to show that
his so-called positive reaction with bacterial proteins, as used
by him in asthmatics, has any bearing upon the bronchial con-
dition or is etiologically diagnostic in any individual case cited.
He has only shown that asthmatics treated with a vaccine were
relieved, irrespective of the reaction obtained with bacterial
protein. His published results must be due to what may be

considered as a non-specific effect obtained by vaccine, thus differentiating the bacterial proteins very sharply from the other foreign proteins concerned in allergy with which non-specific results are not obtained.

Rackemann (5) has supported this thesis of intrinsic bacterial asthmas, but he finds the *non-hemolytic Streptococcus* more prevalent, having isolated this organism in 60 per cent of 40 cases. He used the carbolized bacterial suspension for the intradermal test and he describes positive early reactions occurring within a half hour and characterized by the typical allergic urticarial wheal as well as a late twenty-four-hour reaction with redness, swelling and tenderness. Twenty out of 39 cases tested with autogenous vaccine gave a positive cutaneous reaction, both early and late reactions being used as criteria. In a group of 56 cases he obtained 60.7 per cent of positive reactions, which is considerably higher than Walker's figures of 15.7 per cent, but Rackemann's tests were made by the intradermal method and his cases were selected as probably infective in type. Walker's tests were made by the scratch method and his cases were part of a general group of unselected asthmatics. Rackemann states that "Treatment was successful in fairly close accordance with the presence of a positive skin test."

In this work also the therapeutic results are used as a criterion by which this writer concludes that the cutaneous reaction demonstrates the importance of the bacteria in certain types of asthma. The importance of Rackemann's work lies in the fact that in a majority of his cases he demonstrated the presence of the specific organism in the particular individual. We cannot compare the figures of Rackemann, that is 60.7 per cent of 56 selected cases considered as bacterial asthmas on the basis of a skin test, with his later study (6). These later figures show only 108 cases classed as bacterial asthma in a total of 590 cases, that is 18.3 per cent. In this later work the method of diagnosis of bacterial asthma, that is whether by skin test or not, is not stated.

Is there any way in which this question of the bacterial asthmas can be settled? Two years ago the writer (7) stipulated two

postulates which must be fulfilled in order to establish the proof of the causal relationship between the allergen and the clinical reaction and they hold for bacterial as well as for other substances acting specifically upon hypersensitive man.

1. Hypersensitiveness must be demonstrated by one of the following procedures:
   a. A typical local reaction either cutaneous or ophthalmic must be elicited or
   b. The original allergic manifestation must be reproduced at will on the introduction of the substance, either inhaled, ingested or subcutaneously injected.

2. It must be shown that the individual has come in contact in some way with the suspected substance in order to permit it to act an an etiologic factor.

The writer has studied this subject somewhat from the point of view of these postulates. First, bacterial powders were made of various organisms by the alcohol-ether method described by Walker. The powder was then dissolved in carbolized saline to the point of saturation, filtered clear, and used for intradermal test. The organisms included *Pneumococcus*, types 1, 2, and 3, *Staphylococcus albus* and *aureus*, the latter two each made from at least a dozen strains. Tests were made in a series of fifty cases of bronchial asthma and only two, Case Nos. 2783 and 2879, reacted with a typical immediate urticarial wheal, these reactions being confirmed. The first of these two reacted to the extract of *Staphylococcus albus* and *Pneumococcus* type 1, and the second to *Staphylococcus albus*. Cultures of the nasal and bronchial secretions of the two yielded only a *Streptococcus viridans* in the first case and *Streptococcus viridans* and *Micrococcus catarrhalis* in the second. The *Staphylococcus albus* and *Pneumococcus* type 1 were sought but could not be recovered in either case. The cases in which *Pneumococcus* of the fixed types has been recovered in the sputum have all been tested but never yet shown a positive skin test. There was no suggestion that the bacterial extracts would operate as the other known allergens.

Secondly, it is always possible with extracts of pollens, danders, foods, and drugs, to elicit an immediate constitutional reaction

in a hypersensitive individual by the subcutaneous injection, or, in the case of food and drugs, by the ingestion of the specific allergen in sufficient dosage.   With the bacterial preparations, however, no similar immediate allergic reactions have been obtained in my clinic and I find no record of such in the literature.   The mere increase in the degree of an existing asthma twelve to twenty-four hours later will not suffice as proof.

We have tried by such measures to prove the possible allergic nature of the bacterial reactions and have failed.   Further work might be done and certainly better extracts might be prepared, but from such work we were forced to conclude that the bacteria if they operate as fundamental causative factors in bronchial asthma, do not act as allergens *per se*, but in some way not understood today, and that the cutaneous test with bacterial proteins is of no value in the diagnosis of allergy.

In other words, the conception of a bacterial asthma has been based solely upon analogy and the analogy is not upheld by proof.   "Bacterial Asthma" has become a convenient term by which to designate many of those cases not reacting to the genuine allergens, but such cases should be classed as undiagnosed even though resort be had to vaccine therapy with apparently good results.

Since the diagnosis of bacterial asthma is not as yet susceptible to positive proof and is only arrived at by exclusion the writer has taken the attitude that it is wiser to continue to search for new factors which can be shown to be specific agents in accordance with the postulates laid down.   In this way by increasing the percentage of diagnosed allergic cases the possible cases of bacterial sensitiveness are more accurately separated from the whole group and will lend themselves to more productive study.

2. REFLEX ASTHMA

Most of the present day writers, discussing vasomotor rhinitis and asthma either together or separately, appear to believe that a vasomotor edema of the respiratory mucus membrane with a resulting rhinitis or asthma can be induced by some irritant

acting reflexly upon the membrane. Walker (8) discussing this point says "The causes of symptoms may be classified as mechanical, chemical, odorific and thermal. Among the mechanical causes any kind of dust is the most frequent cause, more especially sweeping dust and hay dust." He further says, "Some of these patients are sensitive to some type of protein which may have rendered their nasal mucus membranes sensitive to these irritants, others are not sensitive to the proteins." This is in part an adoption of Goodale's idea regarding what he has termed olfactory vasomotor rhinitis or pseudohay fever. Goodale (9, 10) mentions the fragrance of certain plants such as lily of the valley, lilac and hyacinth, as excitants of attacks of sneezing as well as asthma, in cases where the tests with the pollens themselves were negative. In other words both these writers conceive that nonspecific irritants acting through a reflex mechanism may be fundamental causes of the asthmatic paroxysm in individuals who are not hypersensitive. This is a return to the idea so well summed up by Osler in the first edition (1892) of his Practice of Medicine. He says

Briefly stated, then, bronchial asthma is a neurotic affection characterized by hyperemia and turgescence of the mucosa of the smaller bronchial tubes and a peculiar exudate of mucin. The attacks may be due to direct irritation of the bronchial mucosa or may be induced reflexly by irritation of the nasal mucosa and, indirectly, too, by reflex influences from stomach, intestines, or genital organs.

The writer (11) has contended that non-specific irritants can only operate in those cases that are specifically hypersensitive. They are never fundamental etiologic factors. To be sure these non-specific irritants do produce attacks of asthma in cases that have not been diagnosed but certainly in no greater number than in the diagnosed group. The further proof of the contention lies in the fact that these non-specific and reflexly acting irritants cease to operate and to be productive of attacks in diagnosed cases where the paroxysms have been absent for a period of time either from removal of the specific cause or after improvement and relief as a result of specific therapy.

It is the special object of this paper to show that the two supposedly reflexly acting mechanical excitants particularly selected by Walker, namely, hay dust and house dust, are genuinely specific factors and that they operate in the specifically hypersensitive individual just as do pollens, animal dander and the other well known allergens that demonstrate their clinical effect after absorption by inhalation.

A. Hay dust

The first case in which hay dust was shown to act specifically is here described.

Case 2207.   A man, forty-five years of age, was seen in March, 1920, when he complained of bronchial asthma.   His father was one of three asthmatic children, in a family of ten children.   The patient's first attack of asthma occurred in 1900 and came on when he was visiting in a farm house to which a barn was attached.   The next attack occurred one year later after he lay down in timothy hay.   Subsequent attacks have come on following the handling of objects that had been packed in timothy hay.   The patient states very positively that he has no trouble (either hay fever or asthma) in the country or elsewhere during the months of May, June and July, when the grasses are in flower.   Tests carried out with the strongest extract of timothy pollen resulted negatively.   Tests carried out later in both the skin and the eye with an extract of the timothy hay itself resulted in both instances in definite positive reactions.   Similar tests made with the same extract upon normal individuals resulted negatively.

Further use of the extract of the timothy hay has verified the specificity of the reactions.   Three of the 327 cases of asthma under consideration were found to be hypersensitive to the substance.   These cases indicate that attacks of asthma on exposure to dusty hay are not to be considered as a reflex effect of a non-specific excitant, as Walker states, but as an expression of a specific allergic reaction.

B. House dust

Following is the history of the case that led to the discovery of the presence in house dust of a specific allergen.

Case 1763, T. F., male, age twenty-six, had had frequent attacks of asthma for fourteen years. He then enlisted in the army in June, 1917, and while stationed in Texas had no trouble at all. He returned home in October for six days and had severe asthma all that time. On his return to Texas the attacks disappeared and he was again free until he returned home in December. He applied for treatment in January, 1918, having been continuously ill and unable to work for a month. He was tested by the intradermal method with extracts of all our then known products including pollens, sachet powders, animal danders, foods, and drugs with negative results. He was advised to use an army cot and air pillow. The attacks were lessened. Several times he slept away from home and was well, but the attacks returned regularly when he stayed at home. Finding all tests negative, a culture of the sputum had been made and an influenza bacillus and *Streptococcus viridans* had been isolated. A vaccine[1] had been made and the patient had been treated for over two months with vaccine injections with no improvement in his condition whatsoever. In April of that year, he was instructed to bring all the dust that could be collected from his room, going over it carefully with a vacuum cleaner. This dust was then extracted just as pollens, orris and other substances are extracted, made sterile by filtration and used for intradermal tests. For the first time in this case very marked positive reactions were obtained in the skin at two sites of test and this was followed in a few moments by the development of a mild constitutional reaction with asthma, coryza, general erythema, and pruritus as the symptoms. With proper dilution of the extract this local reaction could always be elicited.

A number of other cases hitherto undiagnosed gave marked cutaneous reactions with this same dust extract injected intradermally. The patient's home was then investigated. It was a clean, modern house. His mattress was made of long curled horse hair, the pillows were feather; there was only a small rug on the floor. He had been negative to extracts of feathers and to horse hair and continued so on repeated testing. Although it was not possible to discover the exact substance giving the reaction, the presence of a specific airborne factor was demonstrated. The patient then moved to California where he has been entirely free from symptoms.

[1] The culture and vaccine were made by Professor John Torrey of the Department of Hygiene, Cornell Medical School. The examination was made from the fresh specimen.

We were then face to face with a new procedure in the diagnostic study of asthma and with a possible *new but unknown* substance. Efforts to discover the active ingredient in the dust extracts by comparative tests has so far yielded negative results. Of course, in houses in which there is a dog or a cat, the extracts of the dust agree by test but in lesser degree with extracts of the dander of the respective animals. The same holds true of the agreement by test of the dust extracts of rooms in which orris root powders are used with the orris extract itself; and this is the fact in those houses where rabbit hair pillows and mattresses are used. But dust collected from homes where none of these

TABLE 1

Reactions

CASE NUMBER	DUST NO. 1	ORRIS	RICE	WHEAT	TIMOTHY	RAGWEED	HORSE	CAT	DOG	RABBIT	FEATHER	GLUE
2185	+++	0	---	---	0	+++	0	0	0	---	0	+++
2183	+++	+++	---	---	0	+++	0	0	0	0	+++	0
2170	+++	0	0	0	0	0	0	0	+++	+++	---	---
2169	+++	0	0	0	0	+++	+++	+++	0	0	0	0
2165	+++	0	0	0	0	+++	0	0	0	+++	0	---
2196	+++	0	0	0	0	0	---	0	0	0	+++	0
2189	+++	0	0	0	0	0	---	0	0	0	+++	0

+++ = Marked positive.

0 = Negative.

--- = Test not done.

articles can be found also gives an extract which produces a marked positive cutaneous reaction in certain cases, and is still an unidentified factor. The extract does not agree by test with any other known extract used. Table 1 clearly demonstrates this point, and in all of these cases a single extract known as Dust Extract No. 1 was used and all tests were made by the intracutaneous method. The cases in table 1 were selected from those in which marked dust reactions were obtained, but it will be seen that while other extracts are occasionally positive there is no agreement between them and the dust reactions. Many similar cases could be cited, but the table is abbreviated purposely as greater length would not add to the argument.

The preparation of the dust extract is carried out according to the suggestions found in Coca's article in this issue of the Journal of Immunology on the preparation of extracts. In brief, we can say that the dust collected by means of a vacuum cleaner is treated first with ether to remove all fatty substances, and is then extracted with the standard extracting fluid. The extraction is allowed to continue for two or three days when the solution is filtered off, sterilized by filtration through a Berkefeld and put through all the sterility tests. This extract contains nitrogen, probably, in many chemical combinations so that these extracts cannot be standardized satisfactorily by the nitrogen content and the nitrogen determination has only a relative value.

While the chemical studies of these dust extracts have not been completed and are still under way, it can be stated here that by dialysis a considerable quantity of nitrogenous substance appears in the dialysate, but that the dialysate does not contain the reacting substance. When the dust extract is sealed in sterile tubes and heated to 212°F. for thirty minutes there is a diminution in the activity of the extract, but under these conditions no precipitate forms. When heated to boiling in an open vessel so that the carbon dioxide of the extracting fluid is driven off, some precipitation takes place and the extract loses all power of reactivity in a very short time.

The question that naturally arises is, what is the actual or relative importance of this new extract? It can be at once stated that as a diagnostic procedure the testing of dust extracts is of the utmost importance in demonstrating the presence or absence of environmental substances which act on absorption by inhalation, and whether or not this environmental factor is domiciliary or occupational. The writer has always been impressed by the importance of the respiratory tract as the chief path of absorption in all adult asthmatics, and the following table arranged for comparison illustrates this point by the large percentage of cases in the inhalation group. In table 2 the cases of Rackemann and of Walker (12) are arranged for comparison with 327 of the writer's cases of bronchial asthma studied in 1920. The figures for 1921 will be published shortly. Racke-

mann's figures are those supplied by him in a personal communication and are more recent than the figures appearing in his article (6). All those cases, such as bacterial, not diagnosed by means of a positive cutaneous reaction are placed in the general group of "intrinsic asthma" and classed as undiagnosed. In this respect the cases of all three authors are treated in exactly the same manner. It will be noticed that the percentages of cases occurring from pollen, danders, powders, and foods do not make a total of 100 in any of the three writers' statistics. This is due to the fact that many cases are examples of multiple hypersensitiveness and belong in two, or even three or more, groups and are therefore counted more than once. Consequently, in order

TABLE 2

AUTHOR	NUMBER OF CASES	EXTRINSIC CAUSES											INTRINSIC CAUSES
		Absorption by inhalation						By inhalation	Absorption by ingestion				Cases diagnosed	Bacterial asthma. Undiagnosed cases
		Pollens		Animal dander		Vegetable powder			Food		Drugs			
		Number	Per cent	Number	Per cent	Number	Per cent	Per cent	Number	Per cent	Number	Per cent	Per cent	Number	Per cent
Rackemann	590	150	25.0	55	9.0	9	1.5	27.8	19	3.2	0		31.0	408	69.0
Walker..........	400	92	23.0	78	19.5	0		23.0	68	17.0	0		40.0	241	60.0
Cooke..........	327	86	26.0	138	42.0	52	16.0	69.8	12	3.6	4	1.2	73.4	87	26.6

to arrive at the percentage of cases due to substances absorbed by inhalation it has been necessary to take the percentage of cases undiagnosed and by subtraction arrive at the percentage of diagnosed cases. From the percentage of diagnosed cases we subtract the percentage of cases of food reactions and so arrive at the figures for the percentage by inhalation. For example: Among the writer's cases 26.6 per cent are undiagnosed; this means that 73.4 per cent were diagnosed. Only 3.6 per cent of the cases are diagnosed as due to foods, and by subtraction this leaves 69.8 per cent as due to substances absorbed by inhalation. In other words, practically 70 per cent of all the writer's cases studied are due to substances that are conveyed in the form of

dust and that act after absorption by the mucous membranes of the respiratory tract. It deserves some comment that the percentage of cases of the diagnosed group are for Rackemann 31, for Walker 40, and for the writer 73.4 per cent. I believe that these differences can be satisfactorily explained on three grounds. First, the number of routine tests made with extracts of airborne substances. The greater the number of tests the greater the improvement will be in the percentage of positive diagnoses; inevitably this must result in a decrease in the percentage of the so-called bacterial and undiagnosed cases. There are a number of known substances to which Rackemann and Walker appear to attach little or no importance, for either they are not mentioned at all, or only casually mentioned in their writings. For example: Scarcely a day passes at our clinic on which we do not diagnose by positive cutaneous reactions several cases that are hypersensitive to the dander of rabbit and goat. The importance of these reactions lies in the fact that we are able in many cases to demonstrate the presence of these substances in the homes of the individuals reacting. The untreated and unsterilized hair of both rabbit and goat, containing a large amount of nitrogenous substance from the attached dander, is used very extensively, not only in New York but in all large centers of population, by families of foreign birth. The Italians import and use goat hair for pillows and mattresses, while the Germans, Slavs, Poles, and Hungarians, and among these especially the Jews, use rabbit hair for the same purpose. Second, Rackemann and Walker both use the so-called scratch test of Schloss, whereas the writer uses the intracutaneous test which Brown (13), in another article in this issue, shows to be by far the more delicate and the more efficient. Third, the writer uses fluid extracts prepared according to the directions published by Coca (14) in this Journal. These extracts, according to Brown (13) give a greater percentage of positive reactions even when the scratch test is used than do the extracts prepared according to the directions of Wodehouse (15) and made commercially available and used by Rackemann. Walker does not state in his publications what extracts were used by him.

In order to show the importance of the dust extract, actually and relatively, a list is given in table 3 which shows all of the individual substances found to be of diagnostic importance in the 327 cases under consideration.   In this table is also put down the number of times each one of these extracts was found to react as a causative factor, and the dust extract was important in 33 per cent of the entire group.   It is seen that the number of reactions obtained is much larger than the number of cases studied in spite of the fact that 87 were negative to all tests;

TABLE 3

Total number of cases 327

EXTRACT	NUMBER OF CASES REACTING	PER CENT OF CASES	EXTRACT	NUMBER OF CASES REACTING	PER CENT OF CASES
Timothy............	12	3.7	Horse epithelium....	41	12.8
Ragweed............	74	22.0	Cat "	30	9.2
Orris................	47	14.4	Dog "	18	5.5
Rice................	10	3.0	Rabbit "	18	5.5
Wheat..............	8	2.7	Cow "	2	0.6
Corn................	11	3.4	Aspirin.............	3	0.9
Buckwheat..........	11	3.4	Ipecac	1	0.3
Cottonseed..........	2	0.6	Egg	7	2.1
Glue................	4	1.2	Milk	2	0.6
Hay................	3	0.9	Celery	2	0.6
Dust No. 1..........	109	33.0	Peach	1	0.3
Feather.............	84	25.7	Raspberry..........	1	0.3

and this is because a very large percentage are examples of multiple hypersensitiveness.   What has been said in this paper for the importance of dust extracts in asthma obtains in the same way and in the same degree in allergic coryza.

CONCLUSIONS

In this paper it has been shown that the group of substances absorbed by inhalation play a much more important part as specific causative factors of asthma than is generally considered to be the case by other investigators.   Diagnoses arrived at in accordance with this idea may be made with a greater degree of assurance on account of the fact that they are based upon posi-

tive findings and not upon negative findings, as is the case with the so-called bacterial asthma cases, which the writer insists are to be more properly classified as undiagnosed. The new procedure of testing dust extracts has yielded valuable information in that it permits a study of the occupational or domiciliary environment of an asthmatic and establishes a positive diagnosis in certain cases not obtainable by any other means. Further, it has shown the presence of a substance in most house dusts that is in itself an important factor, but the nature and source of which is as yet unknown. The dust of hay, also, may act as a specific allergen and is not to be considered solely as a simple mechanical irritant.

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

V. THE PREPARATION OF FLUID EXTRACTS AND SOLUTIONS FOR USE IN THE DIAGNOSIS AND TREATMENT OF THE ALLERGIES WITH NOTES ON THE COLLECTION OF POLLENS

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From the beginning of his work upon the diagnosis and specific treatment of the allergies (hay fever, asthma, the urticarias), Robert A. Cooke (1, 2) has employed for both the diagnostic and therapeutic injections, fluid extracts of the various materials containing the exciting agents of these conditions. These extracts were originally made with physiological salt solution to which was added the usual percentage of carbolic acid The preparations were standardized according to their nitrogen content. Cooke and also Cooke and Vander Veer, and Vander Veer, have reported their successes in the use of these extracts as diagnostic and therapeutic preparations At the time when these investigators became associated with the writer in the organization that has been established in the New York Hospital for the diagnosis and treatment of the allergies, some of the extracts that had been in use were subject to certain disadvantages. In particular, the pollen extracts were often quite unstable in their activity both as diagnostic agents and as therapeutic material; the extract of feathers, especially chicken feathers, seemed to possess little or no specific activity in cases of known hypersusceptibility to these materials.

The preparation of fluid extracts was undertaken by the writer with the purpose of overcoming the disadvantage mentioned. It was necessary to provide a sterile preparation of not too low

concentration which would remain stable for at least six months. The pursuit of this problem was attended with some serious difficulties which impeded its purpose. One of these difficulties lay in the fact that the sole test object was a human being suffering from a distressing condition, which might or might not be relieved by the therapeutic use of the new kind of protein preparation.

There was, therefore, a natural hesitancy to change from one kind of preparation to another for experimental reasons. Furthermore, the comparison of different preparations by means of the cutaneous or of the ophthalmic reaction is interfered with by the natural difficulty of obtaining consent of suitably sensitive individuals to submit to the experimental injections. The problem was further complicated by the number of the possible factors concerned in the deterioration of the preparations; for example, light, temperature, chemical reaction, quality of glass in which the preparations were stored, and sterility.

Indeed, it must be admitted at the outset that some of these factors have not yet been investigated. It is not known why the preparations in present use satisfy the requirements of potency and relative stability; whether on account of mere sterility or on account of a modification of the composition of the extracting fluid. In any case the preparations about to be described are to be looked upon as merely modifications, perhaps only slight ones, of those employed previously by Cooke and his associates.

While the problem is still under investigation it has been found desirable to make the present preliminary publication on account of the thoroughly successful diagnostic and therapeutic use of which the preparations have been found capable.

In deciding upon the composition of the extracting fluid, the fact has been borne in mind that the exciting agents often reach the sensitive mucous membrane in a dry state. Hence it could be assumed that the active principle in these materials was soluble in a slightly alkaline and saline solution. While it was known that certain vegetable proteins for example, gliadin, are soluble in aqueous media only in the presence of free alkali (sodium hydrate in hundredth normal concentration or stronger),

it was believed that such proteins could have little significance in human hypersensitiveness since they are not soluble in the natural secretions of the mucous membranes. Free alkali was thought to be disadvantageous as an extracting agent on account of its known tendency to denature certain proteins. The desired alkalinity was therefore obtained with the use of sodium bicarbonate. This salt has the desirable faculty of neutralizing both free acid and free alkali and is able, in consequence, to correct any change toward either reaction which they tend to develop in the course of preparation, or during storage.

After some experimentation, the following composition of the extracting fluid was adopted.

Sodium chloride... 0.5 per cent
Sodium bicarbonate.... $NaHCO_3$ in such concentration that 10 cc. of the final fluid equalled about 3 cc. of $\frac{N}{10}$ alkali
Carbolic acid....................In final concentration of 0.4 per cent

The solution was made without the use of heat and with the avoidance of excessive shaking. When it was desired to dilute an extract, or other preparation with this fluid after the original extract had been sterilized, the diluting fluid itself was sterilized by filtration through a sterile Berkefeld filter.

As a general rule this alkaline extracting fluid is used for all dry materials such as the cereals, the danders, the nuts, and the pollens. It is also used for certain vegetables that contain little juice, such as sweet potato, fresh beans, fresh peas and for the meats. The extracting fluid is used as a diluting fluid when dilution of the extract is desired. When the original material contains considerable fluid such as the fruits and most of the vegetables, it is advantageous, in order to avoid too great dilution, to use a "preserving fluid" containing the constituents of the "extracting fluid" in a higher concentration. Such a preserving fluid we have prepared containing 2.5 per cent NaCl, 1.25 per cent $NaHCO_3$,2. per cent carbolic acid. It has been found that many of the fruit juices contain much more than enough acid to neutralize all of the $NaHCO_3$ in the added preserving fluid. We do not know that the resulting partial neutralization of the acid is of any advantage in the preparation of

the acid juices. For several reasons it has not been possible to make an adequate study of the method of preparing these juices. We have used the preserving fluid referred to for both the neutral and the acid juices on the ground of convenience.

The extraction of the dry materials is carried out at room temperature and usually this extraction is continued for forty-eight hours—sometimes for three days. In these conditions the concentrations of carbolic acid (0.4 per cent) in the extracting fluid is not always sufficient to present the multiplication of bacteria in the mixture. This difficulty was met with the use of toluol, which is able not only to prevent bacterial growth but to kill nonspore bearing organisms. When the material to be extracted is in the form of a powder or fine meal it is advantageous to mix the toluol with the powder before adding the extracting fluid as this makes certain that the toluol reaches all of the substance. It also prevents the formation of clumps which are difficult to break up.

If the material contains oily substances which may interfere with the infiltration of the extracting fluid, the oil is first removed with ether which does not denature proteins. We have not found it necessary nor convenient to use a special extraction apparatus such as the familiar Soxhlet apparatus. We have simply mixed the ether with the substance in a sedimenting jar and as soon as sedimentation was complete we have decanted the ether and made further extractions in the same way with additional portions of ether.

As the different materials contain different percentages of oily substances, the number of changes of ether must be varied. With nuts and oily seeds, we have been guided in this respect by making a rough determination of the quantity of oil removed by the successive fractions of ether. This determination was made by evaporating a few cubic centimeters of the etherial extract in a beaker immersed in hot water and noting the quantity of the oily residue. With the pollens the extent of the oil extracted could be judged by the depth of color imparted to the successive portions of ether. Complete extraction of the oily substances has not been found necessary for some materials, such

as the nuts and the pollens. After the extraction of most of the oily substances the ether has been driven off from the material before it was mixed with the extracting fluid.

In most of the extracts and preserved juices a precipitate forms upon standing. As precipitation continues even after the fresh extract has been filtered, it is necessary to wait until the precipitation is complete before carrying out the further steps of the preparation. In the case of some of the vegetables, this precipitation has been found to cause relatively little reduction of the nitrogen content of the extract. On the other hand, the precipitate in the extract of the meats and fish is doubtless wholly proteid. Conceivably some of the exciting agents of the allergy to the original material are in part or entirely lost by this precipitation. We have no evidence to offer on this point. The separation of the precipitate from the fluid extract offers difficulties varying in degree with the different materials. If the precipitate is not too voluminous it can be removed at once with the use of the Sharples centrifuge. This instrument, which is almost indispensable in the preliminary clearing of larger volumes of fluid, is not adapted to the handling of quantities much less than five hundred cubic centimeters. The smaller quantities of extract may be filtered through paper, preliminary to the final sterilizing filtration through Berkefeld or similar filters.

The precipitate that forms in some of the extracts is so voluminous that it is impossible to use the Sharples centrifuge for its removal. In such a case, a partial separation can usually be effected with the use of a fine mesh towel laid over a sieve. As the precipitate tends to form an impervious mat upon the cloth, it is necessary at intervals to scrape off the collected precipitate with a large spoon. During the period in which the sedimentation is taking place in the extract it is advisable to keep the fluid covered with a shallow layer of toluol. To prevent evaporation of the toluol the container should be tightly stoppered. Many of the extracts are found to be sterile after having stood for three days or longer under toluol. Advantage has been taken of this circumstance in the preparation of some protein substances, particularly the isolated globulins, which are not at all

or with difficulty filterable through the Berkefeld type of filter. However, sterilization of the extract has been secured for much the greater part by Berkefeld filtration.

The filtering flasks were sterilized as usual with dry heat. The filters were sterilized by one exposure in the autoclave to steam under a pressure of fifteen pounds for fifteen minutes. The connecting perforated rubber stoppers were boiled for ten minutes in 5 per cent carbolic acid and again for ten minutes in water. The main stock of the filtered extract was stored in sterile 16-ounce bottles. For clinical purposes extracts are distributed in vaccine bottles or homeopathic vials, which are capped with "No-air" stoppers. The greater part of the stopper must be cut off with scissors so that the cap may be more readily punctured by the syringe needle in removing the contents of the bottle for test purposes. The bottles and caps are sterilized separately by boiling for twenty minutes in plain water. The caps are held firmly on the vials with the use of narrow rubber bands.

Sterility tests have been made by introducing about 0.25 cc. of a filtered preparation at the bottom of a 12- to 15-cm. column of neutral nutrient agar containing 1 per cent of dextrose. Another portion of the preparation was deposited at the surface of the agar in the same tube. The agar was quickly solidified by placing the tube in cold water. The nitrogen content of all the preparations was determined by the Kjeldahl method and generally adjusted by dilution to 0.5 mgm. or less per cubic centimeter. Further dilutions of these "stock solutions" were made for diagnostic and therapeutic use.

It is not in the province of this article to discuss the dosage of the different preparations nor the dangers attending their use. These questions are considered in the various publications of Cooke, Vander Veer and Brown. It may be stated here, however, that the nitrogen content of the usual dilutions of all the pollen extracts was: 0.1, 0.05, 0.01, 0.005 and 0.001 mgm. With many of the vegetables and fruits it was thought advantageous to leave the nitrogen content at its original concentration.

While there are some general principles that apply to all of the members in each of the several groups of materials, the method

employed for each group must be modified to meet certain peculiarities of some of the members of the group.   It is intended to refer to some of these peculiarities, although it is not possible in the limits of this communication to describe the procedure followed in each of the individual extracts that have been prepared.   It will be useful to describe in some detail the method employed in several instances under each group.

Dry materials

Whole wheat flour: One kilogram of the flour was thoroughly mixed with 150 cc. of toluol and then with 2200 cc. of extracting fluid.   After twenty-four hours at room temperature in which the mixture was once thoroughly stirred up the supernatant fluid was drawn off into a filtering flask with the use of negative pressure.   With the sediment 2000 cc. of fresh extracting fluid were mixed and after a further period of twenty-four hours the supernatant fluid was drawn off as before and mixed with the first extract.   Three liters of fluid were thus obtained. A slight sediment settled in the fluid over night, which was not greatly increased in the next few days.   The supernatant fluid was filtered through a Berkefeld filter.   Filtration was slow, but not so slow as it was with an extract of another sample of whole wheat flour which had been prepared previously.   One cubic centimeter of the filtrate was found to contain 175 mgm. of nitrogen.

Rice polish:[1] 1½ pounds of rice meal were mixed with 150 cc. of toluol and this mixture was stirred well first with one liter of extracting fluid; to this mixture was then added a second liter of extracting fluid; the mixture was left at room temperature until the following day when 300 cc. of supernatant fluid were drawn into a filtering flask.   750 cc. of extracting fluid were mixed with the sediment and on the following day an additional 750 cc. of supernatant fluid were obtained and mixed with the first extract.   After a first filtration through the Berkefeld filter the nitrogen content per cubic centimeter was 1.4 mgm.   A slight

[1] A generous quantity of this material, which is also called rice meal, was kindly donated by the Louisiana State Rice Milling Co., 100 Hudson Street, New York City.   It has been assumed that the allergen of rice is a protein which is the same in all parts of the grain.   In accordance with this idea the rice preparation has been made from the materials ("rice bran" and "rice meal"), which are removed from the grain in the process of polishing, because these materials contain about 14 per cent of protein.

precipitation made a second filtration necessary. The nitrogen content after this final filtration was practically the same as it was after the previous filtration.

Dry "navy" bean: The dry beans were ground in a meat chopper and the coarse meal was then ground in a coffee mill. The powder was mixed with toluol and extracting fluid. After twenty-four hours the fluid was pressed out through a clean towel and centrifuged in a Sharples centrifuge. The extract was covered with toluol in a tightly stoppered flask and allowed to stand at room temperature. After five days a voluminous precipitate had formed and settled. The precipitate was removed in the Sharples centrifuge. After a further five days under toluol a second precipitate had formed which was likewise removed by centrifugation. Precipitation continued for several months. A clear extract was obtained at the end of six months in which no further precipitation had taken place one month later. One cubic centimeter of extract contained 5 mgm. of nitrogen.

Horse dander: A quantity of dander obtained by currying and containing few hairs was mixed with three volumes of ether by stirring. After the sediment had completely settled the ether was decanted and discarded. The material was again extracted with another equal portion of ether. The ether was completely removed from the sediment by stirring the latter in a beaker which was immersed in hot water (50 to 60°C.). Fifty grams of the resultant material were mixed with 100 cc. of toluol and then with 1000 cc. of extracting fluid. The mixture was allowed to stand over night in a stoppered flask. During this period the mixture was shaken once. On the following morning the entire mixture was shaken up and thrown on a hardened filter paper. Eight hundred cubic centimeters of a clear brown filtrate were obtained after six hours, this quantity being increased to 800 cc. over night. The filtrate was immediately filtered through a Berkefeld filter. One cubic centimeter of the filtrate contained 0.5 mgm. of nitrogen. The filtrate remained clear.

Pollen: 90 grams of ragweed pollen were treated with ether in the same way as was the horse dander until the decanted ether showed only a slight yellow color. Four or five extractions suffice for ragweed pollen. The grass pollens contain less oil than the ragweed pollen. After the ether had been removed from the pollen as in the case of the dander, the entire quantity of pollen was mixed with 2700 cc. of extracting fluid. The mixture was covered with toluol as usual. After four days, during which time the sediment was shaken up once or

twice daily the supernatant fluid was decanted and the sediment was mixed with a second portion of 450 cc. of extracting fluid. As soon as the sediment had settled the supernatant fluid was decanted and mixed with the first portion. The combined decanted fluid, amounting to 3000 cc. was filtered through a Berkefeld filter. One cubic centimeter of filtrate contained 0.3 mgm. of nitrogen. The filtrate remained clear. One cubic centimeter of an extract of timothy pollen made in the same way contained 0.42 mgm. of nitrogen.

Some idea of the rate of deterioration of the pollen extracts was obtained in the following experience:

Four different preparations of the extract of timothy pollen were made with the extracting fluid in the manner above described.

Preparation Ta was made March 4, 1921 and kept in the ice-box.

Preparation Tb was made December 9, 1921.

Preparation Tc was made November 10, 1920, and kept at room temperature (15 to 32°C.).

Preparation Td was made February 12, 1921, and kept at room temperature.

The nitrogen content of the four preparations per cubic centimeter was as follows: Ta—0.4 mgm., Tb—0.36 mgm., Tc—0.28 mgm., and Td—0.336 mgm. All of the older preparations had been preserved in their original concentration. A few days after preparation Tb was made, the four preparations were diluted to an equal nitrogen content in three different concentrations and compared in the clinic as to their specific activity by intradermal injection in an individual subject to early hay fever. The results are shown in table 1.

These results indicate that the pollen extracts deteriorate somewhat in nine months if they are kept in the ice-box and that this deterioration is considerably greater if the extracts are kept at room temperature.

Feathers: Feathers are washed once with enough ether to wet them well. The ether is wrung out by hand and the feathers are spread upon clean newspapers to dry. The removal of the ether is hastened by occasionally turning over the mass of feathers and by compressing

them.   The feathers from which most of the oil has been thus removed are passed in successive batches through 2 one-liter portions of extracting fluid, each batch being allowed to remain about ten minutes in each portion of fluid.   Naturally the successive batches of feathers carry a certain quantity of fluid with them after the immersion, and this circumstance places a limit on the quantity of feathers that can be treated in this way by the two liters of extracting fluid.   On the other hand, the more feathers used the greater will be the concentration of the extractable protein in the resulting extract.   After the last batch of feathers has been carried through, the two portions of fluid are combined and cleared in the Sharples centrifuge.   As precipitation sometimes takes place in the extracts of feathers it is well to allow them to stand for a few weeks under toluol before the final Berkefeld filtration.

Hair and wool are treated in the same way as feathers.

TABLE 1

Showing the relative specific activity of the different extracts of timothy pollen on intradermal injection

	0.001*	0.005	0.01
Ta.............................	++	+++	++++
Tb.............................	+++	++++	++++
Tc.............................		+±	+++±
Td.............................		++	+++±

* Milligrams of nitrogen per cubic centimeter.

Moist materials

Moist materials, from which little or no juice can be expressed by hand, such as meats, sweet potato, green pea, turnip, cauliflower, and lima bean, are passed twice through a meat chopper, mixed with extracting fluid and covered with a thin layer of toluol.   The extraction is generally interrupted after twenty-four hours and the fluid is obtained by pressing the mixture in a stout towel by hand.   The amount of extracting fluid used varies with the nitrogen content.   For the meat three or four volumes of the fluid (figured on the weight of the material) are used.   For the vegetables, one or two volumes.

Some of the shell fish yield a juice in addition to the meat.   To the juice one-quarter of its volume of preserving fluid is added.   With the chopped meats three or four volumes of extracting fluid are mixed. These two portions are then thrown together and allowed to stand under

toluol for at least two weeks.   With some of the fruits and vegetables that can be peeled, such as peach, tomato, orange, lemon, and grape fruit, it is advantageous to make separate preparations of the pulp and the peel.   The pulp in such case is carefully separated from the remainder of the fruit and the juice is obtained from it by squeezing through a towel; the peel is ground in a meat chopper and mixed with a quantity (not too great) of extracting fluid.   Juices of fruit and of some of the vegetables, such as string bean, celery, cabbage, lettuce, spinach, cucumber, and white potato, are mixed with one-quarter of its volume of preserving fluid and allowed to stand in well stoppered containers under toluol with which the fluid should be once thoroughly shaken.

Miscellaneous preparations

In general, heat has been avoided in the preparation of the extracts, although according to Wodehouse this precaution is not necessary in the case of some of the vegetables.   In the preparation of fish glue, the glue has been diluted with ten times its weight of 0.5 per cent sodium chloride.   The diluted material has been sterilized by boiling.

Egg preparations

As in the early experiments of Schloss, different proteins of the egg have been isolated for separate testing.   The first step in all the methods of separation of the egg white proteins consists in dissolving the egg white in water.   This process is expedited by forcing the whites through a moist towel into the diluting fluid.   The eggs used for the purpose were strictly fresh (not more than a few days old).   Euglobulin was obtained by saturating a 30 per cent solution of egg white with sodium chloride.   The globulin was purified by repeated precipitation.   The final precipitate was dialyzed against 1 per cent sodium chloride in the presence of toluol.   The diluted material was kept under toluol until the solution was found to be sterilized.   This method of sterilization was made necessary by the fact that the globulin could not be filtered through the ordinary Berkefeld filter.   The use of the preparation was discontinued because no way was found to prevent continued precipitation of the substance.

Pseudoglobulin, crystalline egg albumin and ovo-mucoid were separated in the usual way, except the final stages of precipitating and washing the coagulable proteins with alcohol.   This step was omitted because of the resulting denaturing of the proteins.   These proteins can be sterilized by filtration.

In carrying out the cutaneous test upon individuals believed to be sensitive to egg white proteins it is convenient to make a preliminary test with the whole egg white. This procedure, however, suffers from the disadvantage that the several component proteins of egg white are not present in equal concentration. At the suggestion of Dr. Cooke we have attempted to overcome this difficulty by mixing equal volumes of solutions of globulin, albumin, and ovo-mucoid. These solutions contain the respective isolated proteins in the same concentration; namely, 1.5 mgm. of nitrogen per cubic centimeter. The globulin had been separated by repeated precipitation with half saturated ammonium sulphate. This preparation no doubt contained albumin. It included both euglobulin and pseudoglobulin. The ammonium sulphate had been removed by dialyzing against changes of 1 per cent sodium chloride. Toluol was used as an antiseptic during the dialysis. The albumin fraction was obtained from the filtrate after the separation of the globulin by the addition of a little acetic acid and by increasing the $(NH_4)_2SO_4$ saturation to a little over 7 per cent. Both crystalline and non-crystalline albumin were thus included in this fraction.

Dilutions of the egg and milk proteins have been made with 0.4 per cent carbolic acid in 1 per cent sodium chloride. This diluting fluid was sterilized by Berkefeld filtration.

Carbolic acid should not be mixed in concentrated solutions, such as 5 per cent, with the diluted egg proteins, as this causes precipitation of some of the proteins.

Yolk proteins: For the separation of ovo-vitellin we have used the method of Levene and Alsberg, up to the point of extraction with alcohol. This latter step was omitted. The final precipitate was dissolved in 10 per cent sodium chloride and mixed with an equal volume of distilled water which was added slowly with constant stirring. Since the substance is insoluble in physiological saline solutions it is possible that even a skin potentially sensitive to it would fail to react to its injection. No diagnostic reactions have been obtained with the preparation. Yolk proteins soluble in physiological salt solution.

The following method has been used to obtain yolk proteins that are soluble in physiological saline solution:

The yolks were separated from the whites and placed in 1 per cent sodium chloride solution. Each yolk was lifted out of the fluid in a large spoon and the chalazae and film of egg white were removed with the use of a soft towel and scissors.

After a further rinsing in a large dish of 1 per cent sodium chloride solution each yolk was again lifted out of the fluid on the spoon and the surface was dried with a towel. The yolks were broken up in a large separatory funnel and treated with several portions of ether, then with about $\frac{2}{3}$ to $\frac{3}{4}$ volume of 10 per cent sodium chloride. To the resulting solution were added 5 to 6 volumes of distilled water which precipitated the ovo-vitellin. The precipitate was removed by paper filtration. The filtrate was acidified with a few drops of 40 per cent acetic acid and mixed with somewhat more than an equal volume of saturated ammonium sulphate. The resulting precipitate was collected on a paper filter, placed in a dialyzing bag (fish skin) and dialyzed against changes of 1 per cent sodium chloride until no trace of sulphate remained in it. Toluol was depended upon for sterilization.

Milk proteins: Casein is precipitated as usual with acetic acid and separated from the fluid by straining through a stout towel. The towel containing the casein is immersed in changes of water in which the casein is thoroughly washed. With the aid of a pestle, the casein is forced through a wire kitchen strainer, while an assistant keeps the casein well moistened with distilled water. To this suspension of the precipitated casein in water 4 per cent sodium hydroxide is slowly added, while the mixture is continually stirred. The addition of the alkali is continued until early all of the casein is dissolved. Stirring is continued some time beyond this point and if some casein still remains undissolved the solution will be neutral to litmus.

The mixture is filtered through paper and the filtrate is kept under toluol. Before filtration through a Berkefeld filter, the solution is further diluted with 0.4 per cent carbolic acid in 1 per cent sodium chloride to a nitrogen content of 2 mgm. per cubic centimeter. After filtration, the nitrogen content per cubic centimeter is further reduced to 1 mgm.

From the original acetic acid filtrate the other proteins are precipitated with the addition of $1\frac{1}{2}$ volumes of saturated ammonium sulphate and the precipitate, after its collection on a paper filter is freed of the ammonium salt by dialysis against changes of 1 per cent sodium chloride.

COLLECTION OF POLLENS

In the collection of pollens, two principal requirements have been laid down. The first of these has been to obtain the pollen as free as possible from other material (dust from the soil and

from other parts of the plant); the second equally important requirement is that all moisture be eliminated from the collected pollen before it is stored. The method of collection is different with the different groups of plants; that for the grasses is as follows: the heads are cut off with scissors and spread, in not too thick layer, upon strips of glazed paper.

In order to obtain the pollen as free from extraneous matter as possible it is important to shake the collected heads vigorously over a sieve or coarse wire netting before spreading them on the paper. The room used for this purpose should be warm, dry, well lighted and protected from dust. The attic rooms of the ordinary house are generally suitable. Ordinarily the greater part of the pollen obtainable in this way drops out of the heads within twenty-four hours. It has been found, however, by transferring the heads to a fresh sheet of paper at the end of twenty-four hours, that an additional quantity of pollen can be obtained in the second twenty-four hours. For this reason alone it seemed profitable to leave the heads on the glazed paper for at least forty-eight hours. A more important advantage, however, is offered by the observance of this longer period. This advantage is the more thorough drying of the pollen which is so necessary to its proper preservation. Pollen that has been stored in tightly stoppered bottles without first being allowed to dry tends after a time to form clumps and may even become united in a single mass, perhaps under the influence of bacterial growth. On the other hand, if it is sufficiently dried, the material remains indefinitely in the form of a fine yellow powder.

If the pollen in this condition is protected from moisture as in a tightly closed fruit jar, the activity of the extracts made from it after at least a year's standing corresponds as in the beginning with the nitrogen content. There is thus no evidence of deterioration.

At the end of forty-eight hours the heads are taken up from the paper and rubbed in a large wire sieve in order to obtain most of the pollen that still adheres to them. The pollen together with the dried flowers of the grass, which likewise have dropped off or are rubbed off in the sieve, are collected in one place by raising

the ends of the paper strips and tapping the under surface of the paper. The whole mass is then emptied into a clean enameled pan. Most of the dried flowers can be removed from this mass by preliminary sifting with a fine domestic sieve of about four inches diameter. The final sifting should be carried out with a 200-mesh copper wire sieve with the use of a soft, fine, hair brush of one to one and a half inch breadth.

The season for collection is different with the different grasses. That for the most important member of this group, the timothy grass, begins in the neighborhood of New York City about June 20 and ends practically July 6. The heads are not ready for picking until some stamens have appeared on them. The most favorable time of day for collection is in the late afternoon and evening. We have an impression that the yield of pollen is somewhat greater when the heads are collected soon after a shower.

The season for collecting ragweed pollen begins about August 20 and ends about September 5. The method used for the grasses is not applicable here. It was thought necessary, therefore, to shake off the pollen as it ripens naturally in the field.

The pollen of a single plant does not ripen all at one time but over a period of a week or longer. Several of the sessile anthers on a single bract open each day and the contained pollen is released. This process takes place usually in the morning soon after the sunlight has reached the plant. It is profitable to begin the collection as soon as the anthers have opened because after this has taken place the pollen begins to drop out by its own weight or is carried away by the wind, which generally begins to blow after sunrise. The pollen is collected by bending the tops of the plants down so that they can be inserted into the collecting bags and gently shaken. The pollen is readily loosened in this way. Too vigorous shaking loosens the pollen on other parts of the same plant. It requires some dexterity to get the tops into the bag before the pollen has been jarred off. The bags are made of the same glazed paper as was used for the collection of the timothy pollen, and they measure about 11 by 11 inches.

While the foregoing method of collecting ragweed pollen will always be useful for small-scale collection, it will be superseded for collection on a large scale by the method about to be described.

After the bracts have begun to open the stems that bear them are gathered and allowed to dry. When the bracts are thoroughly dried they are stripped off the stem and ground in a mortar or in a wooden ball mill in order to loosen the pollen. The ground mass contains not only the pollen but also other portions of the bracts. It was observed that the pollen sediments rapidly in ether but floats in carbon tetrachloride and it was suggested by Olin Deibert of this division that in a mixture of these two fluids the pollen alone might float whereas most of the other parts of the plant might sink. After some experimentation by Deibert a mixture of 75 parts of carbon tetrachloride and 25 parts of ether was found to effect the desired result. The pollen recovered by this method may be washed with two changes of ether, then dried and sifted in a 200-mesh wire sieve and preserved in tightly stoppered bottles.

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

VI. DERMATITIS VENENATA

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Among the various forms of human hypersensitiveness, the susceptibility to "poison ivy" presents certain peculiarities which make it particularly interesting. One of these peculiarities is the constancy and characteristic features of the lesion. These features are the vesicular form, the erythema and the pruritus. Some preliminary observations by my colleagues, Drs. Cooke and Coca, seemed to indicate two other striking peculiarities of this condition. Cooke's experiences (unpublished) had resulted in the impression that a relatively high percentage of adults possess a sensitiveness to the active principle of "poison ivy" as shown by experimental contact carried out with a method which will be presently described. A short series of tests carried out by Coca (1) with the same method seemed to indicate that a considerable difference of susceptibility to "poison ivy" existed between adults and children under five years of age. Both of these impressions demanded further investigation before a final decision regarding them could be reached. Such an investigation was undertaken and the results of it are here presented.

There is considerable confusion in the literature as to the nomenclature of "poison ivy." In the present paper we have employed the term *Toxicodendron radicans* (L.), to designate "poison ivy," "climbing ivy" or "three-leaved ivy," thus following the classification of Kuntze as adopted by the United States Herbarium at Washington, D. C., the New York Botanical Garden, and other institutions. This classification appears

to be the most logical, since the word "Rhus," the term used hitherto for the genus, is applied to the members of the Sumac family, that are not poisonous, being replaced by the genus term "Toxicodendron" (Greek, "poison tree"). Under this system of terminology, *Rhus toxicodendron* L. (*Rhus quercifolia* Steud.) "poison oak," eastern species, becomes *Toxicodendron toxicodendron* (L.) Britton. *Rhus vernix* (L.), *Rhus venenata* D. C., "poison sumac," "swamp sumac" or "poison-wood," a plant that is not a true sumac, becomes *Toxicodendron vernix* (L.) Kuntze. We are indebted to Mr. Percy Wilson of the New York Botanical Garden for his assistance in the problem of proper terminology.

Many explanations have been offered as to the specific cause of Toxicodendron poisoning.[1] Burrill (2) believed that poisoning of *Rhus toxicodendron* [*Toxicodendron radicans* (L.), Kuntze,] was due to bacterial growths, but later (3) admitted that his hypothesis had not been proven. Khittel, as quoted by Warren (4), claimed to have found a volatile alkaloid as the cause, while Maisch (5) isolated a volatile acid which he called "toxicodendric acid," and to which he ascribed properties similar to formic and acetic acids. Pfaff (6) proved that toxicodendric acid was in reality acetic acid, and stated that the toxic agent was a non-volatile oily substance which he named "toxicodendrol." Acree and Syme (7) considered the poisonous principle to be a glucoside, yielding rhamnose, fisetin and gallic acid upon boiling with dilute acids. Warren (4), working upon the poisonous principle of *Rhus vernix* [*Toxicodendron vernix* (L.), Kuntze], and McNair (8), studying the poisonous principle of *Rhus diversiloba*, [*Toxicodendron diversilobum* (T. and G.), Greene] were unable to obtain results comparable to those of Acree and Syme. Warren and McNair isolated an acid resin. Stevens and Warren (9) consider the active principle of *Rhus vernix* [*Toxicodendron vernix* (L.), Kuntze] to be a resin which is

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[1] The term poisoning is used in this paper as a convenient designation of *Dermatitis venenata* due to "poison ivy," although this condition is obviously not a true poisoning, since the active principle by injection has no effect upon lower animals.

a "clear, amber, oily, red, non-volatile liquid" and "a powerful escharotic."

With the use of a 95 per cent ethyl alcoholic extract of the fresh leaves of *Toxicodendron radicans*, Cooke (10) was able, in 1916, to reproduce the typical vesicular lesion of "poison ivy" in susceptible individuals. Cooke employed the following method of applying the extract for test purposes.

The extract was made by mixing 95 per cent ethyl alcohol with fresh leaves of *Toxicodendron radicans*, which first may be run through a meat chopper. This mixture was allowed to stand for a few days, and the clear extract was obtained by paper filtration. A deposit of some resinous material upon the wall of the flask indicated that the extract represented a saturated solution of the active principle. In our experience, such an extract shows no tendency to a lessening of its activity within at least six or eight months. The extract employed in this investigation was at no time more than four months old.

In the center of the gummed surface of a square of adhesive tape, 5 by 5 cm., there was placed a bit of white blotting paper 0.5 by 0.5 cm., which was then saturated with the alcoholic extract of *Toxicodendron radicans*. The gummed surface of the square of adhesive was then applied to the skin, thus bringing the bit of blotting paper, while still saturated with the alcoholic extract, into intimate contact with the epidermis. We have termed this the "patch test." The flexor surface of the forearm was chosen because of the more delicate texture of the skin of this region, and because of its accessibility. There was no previous preparation of the surface to be tested. After a period of three days the patch was removed, and the area was washed, first with ether to take away any remaining portions of the adhesive, and then with alcohol to remove all traces of the active principle. The skin was inspected for the vesicular lesion typical of ivy poisoning. Every forearm that had been tested was examined twice a week, and differences in the findings were noted. A reaction was considered positive when a typical vesicular lesion of poison ivy was reproduced beneath the patch. No case was considered positive unless there was vesiculation with

erythema and local itching.   Plus signs, with the usual interpretation, are employed in the tables to describe the degree of reaction.

The solid residue of a chloroform extract was also employed. This material was applied directly to the skin over an area about 0.5 cm. in diameter.   This area was covered with a square of adhesive tape to the center of the gummed surface of which was placed a square of glazed paper about 1 by 1 cm. to prevent absorption of the residue by the adhesive material.

TABLE 1

Comparison of the intracutaneous and patch methods

NUM-BER	SEX	INITIALS	DAY OF OBSERVATION									
			Third		Seventh		Tenth		Fourteenth		Seventeenth	
			Intracutaneous	Patch	Intracutaneous	Patch	Intracutaneous	Patch	Intracutaneous	Patch	Intracutaneous	Patch
66852	F	S. B.	0	+	0	++	0	++	0	++	0	++
67001	F	H. L.	0	+	0	+	0	+	0	+	0	+
72295	F	I. G.	0	++	0	++						
72294	M	A. K.	0	++++	0	++++	0	++++	0	++++	0	++++
69407	F	C. S.	0	+++	0	+++	0	+++	0	+++	0	+++
71003	F	T. D.	0	0	0	0	0	0	0	+	0	+
73603	F	G. O.	0	+++	0	+++	0	+++	0			

At the beginning of this study a comparison was made between the intracutaneous and the patch methods of testing.   The usual intracutaneous method was employed in performing these tests.   A fine hyperdermic needle was introduced into the layers of the skin, and a small wheal was produced by the injection of about 0.05 cc. of the alcoholic extract of *Toxicodendron radicans*. A control of 95 per cent ethyl alcohol was injected in each case.

It was found in a selected series of seven cases (table 1), that whereas all the cases were positive to the patch test, three being markedly so, in none was there a positive result with the intracutaneous method.   In a series of thirty cases, using the ender-

mal (intracutaneous) tests, Strickler (11) asserts that "a positive reaction is indicated by a papule, redness and tenderness at the site of injection," at the end of forty-eight hours, and claims to be able to differentiate between ivy and sumach poisoning with this test. Strickler mentions a list of thirty cases which were successfully diagnosed by this method. His method of preparing the poison ivy extract to be used in the tests, consists of "gathering the fresh leaves of poison ivy and extracting with absolute alcohol, filtering and precipitating. The precipitate is dried and extracted in Soxhlet extractors for ten hours. The extract obtained is dried at low temperature. The toxin is carefully weighed, and dissolved in absolute alcohol, to which a certain amount of sterile water is added to make it non-irritating." This is apparently Syme's (12) method. In our experience the injection of the alcohol alone produced the same effect as did the injection of the extract itself. In both instances, only a papule about 0.5 cm. in diameter with erythema was produced, a lesion quite different from the vesicular lesion of *Dermatitis venenata*. Hence, we considered the intracutaneous test of no value.

DIFFERENCES IN INCUBATION PERIODS

One of the outstanding features of ivy poisoning is the incubation period. In even the most sensitive individuals this feature is present. In the published records of the clinical manifestations of ivy poisoning, there are indications of a difference in the incubation period of this condition in different individuals, but this point has not been systematically investigated. McNair (13) notes that "within twenty-four hours after exposure (to Rhus poisoning) patients frequently break out with a rash. This latent period or period of incubation, is dependent on the slow diffusibility of the poison into and within the skin, as well as in the predisposition of the patient." White (14) has never seen ivy poisoning develop clinically after the fifth day of incubation and in his experience the lesions usually appear within twenty-four to forty-eight hours. Schamberg (15) also thinks that twenty-four to forty-eight hours is the usual clinical incubation period.

As the individuals that were used in the present investigation were kept under continued observation, it was possible to study systematically the differences in the period of incubation. The incubation period was found in some instances to be unexpectedly prolonged. In one individual, in fact, no effect was observed until the twenty-fourth day when a one plus reaction developed at the site of the patch test. This reaction consisted of an area of erythema in which were several elevations not distinctly vesicular. There was definite itching. In the absence of characteristic vesicles there may be some doubt as to the specific nature of this lesion.

The tested individuals have been divided into two groups. Group 1 is composed of those individuals that were observed for a minimum of ten days. The results of the tests of these cases are presented in table 2. Group 2 is composed of those individuals that were observed for only seven days. The results of the tests upon these individuals are shown in table 3. No case was included in either group that was not seen on each consecutive observation day. No cases were recorded that were not observed for at least two consecutive observation days.

In group 1 there are 80 cases, 28 (or 35 per cent) of which were found to be insusceptible to the tests during the entire observation period of 10 to 17 days. These 28 cases were not included in the table (table 2), as they had no incubation period and add no information. If all of the 80 cases had been judged by the results recorded on the third day, 48 cases (or 60 per cent) would have been classed as negative, an error of 25 per cent. If all of the cases had been judged by the third and the seventh day readings only, 40 cases (or 50 per cent) would have been classed as negative, an error of 15 per cent. If these cases had been judged by the third, fifth and seventh day readings, 33 cases (or 41 per cent) would have been judged negative, an error of 6 per cent. From these figures it is seen that the degree of error in the determination of susceptibility by this method drops from 25 per cent on the third day of observation, to 6 per cent on the tenth day of observation. This emphasizes the importance of not judging the reaction from a patch test until after a ten day observation period.

TABLE 2

Group 1. Cases arranged according to length of incubation period

CHART NUMBER	AGE	SEX	INITIALS	Third	Seventh	Tenth	Fourteenth	Seventeenth	Twenty-first	Twenty-fourth
						DAY OF OBSERVATION				
72543	18	M	J. C.	0	0	0	0	0	0	+
76625	48	F	R. K.	0	0	0	+			
70432	21	F	E. K.	0	0	0	+	+	+	
71737	17	F	L. W.	0	0	0	+	+		
70745	42	M	P. V.	0	0	0	+			
70137	44	F	A. B.	0	0	+	+	+		
65139	14	M	A. P.	0	0	+	+	+		
74534	47	M	J. K.	0	0	+	+	0		
66997	10	F	C. B.	0	0	+	+	+		
70728	26	F	E. K.	0	0	+	+	+		
73666	35	M	A. D.	0	0	+	+			
73063	40	M	S. N.	0	0	+	+			
70531	20	M	M. H.	0	0	+	+			
75277	29	M	M. P.	0	+	+	+	+		
71110	45	F	M. G.	0	+	+	+	+		
71090	49	F	M. L.	0	+	+	+	+		
69954	40	F	C. G.	0	+	+	+	+++		
65417	27	M	A. J.	0	+	+	+	+		
70255	31	M	S. S.	0	+	+	+	0		
66107	62	M	L. M.	0	+	0	0			
69844	18	M	E. G.	++++	++++	++++	+			
72294	37	M	A. K.	++++	++++	++++	++++	++++		
65423	8	M	F. B.	+++	++	+	+	+		
61220	24	M	W. M.	+++	+++	++	++	++		
65951	66	M	C. T.	+++	+++	+++	++++	++++		
69407	10	F	C. S.	+++	+++	+++	+++	+++		
71423	20	M	M. M.	+++	+++	+++	++	++		
70256	31	M	P. K.	++	++	++	++	++		
69154	51	F	L. B.	++	+	+	+	+		
75040	45	M	C. L.	++	++	+++	++	++		
66852	44	F	S. B.	++	++	++	++	++		
67001	26	F	H. L.	++	++	+	+	+		
70827	44	F	H. N.	++	++	++	+	+		
62424	40	M	M. M.	++	++	+	+	+		
66616	40	F	I. F.	++	++	+	+	+		
73507	40	F	J. S.	++	+	+	+	0		
62626	12	M	A. O.	++	++	++	+			
73906	30	M	G. B.	++	+	+	+	+		

TABLE 2—*Continued*

CHART NUMBER	AGE	SEX	INITIALS	DAY OF OBSERVATION						
				Third	Seventh	Tenth	Fourteenth	Seventeenth	Twenty-first	Twenty-fourth
63620	10	F	C. R.	+	+	+	+	+		
15660	12	F	L. S.	+	+	0	0	0		
74123	45	F	J. L.	+	+	+	+	+		
66103	22	M	W. S.	+	+	+	++	+		
73514	3	F	M. R.	+	+	+	+	+		
6940	34	M	R. A.	+	+	+	+	+		
67508	28	F	A. M.	+	+	0	0	0		
69572	26	F	E. D.	+	+	+	+	+		
73504	36	F	I. S.	+	0	0	0	0		
71623	28	M	M. S.	+	+	+	+	+		
196	43	F	F. P.	+	+	+				
71615	33	M	J. K.	+	+	+	+++	+++		
65656	60	M	P. C.	+	+	0	0	0		
71827	44	F	H. M.	+	+	+	+			

In group 2 (table 3) there are 24 cases, 13 or 54 per cent of which are negative when classed by the reaction that had developed by the third day only. When judged by both the third and the seventh day observations, 10 or 46 per cent were negative. It will be seen that the results from the third day observations and from the third and seventh day observations in groups 1 and 2 are very similar.

DIFFERENCES IN SUSCEPTIBILITY

It is evident in the results of the tests in all of the tables, that the different individuals are not equally susceptible. In fact, every degree of susceptibility was observed from the least to the greatest. All of the markedly reacting cases had developed the typical lesion by the third observation day. Where the incubation period was longer than three days, the degree of the local test reaction was slight, being one plus in every instance. In three of the most susceptible cases, in addition to the typical lesion produced beneath the patch, lesions upon the arms, hands

and ankles, with general pruritus, had developed within twenty-four hours, that is, before the patch was removed.   Close questioning had revealed that these individuals had not been exposed to poison ivy other than by the patch tests.   In these individuals, the adhesive plaster was still in place upon the third day, and the area was washed with ether immediately upon its

TABLE 3

Group 2. List of cases observed for 7 days only

CASE NUMBER	AGE	SEX	INITIALS	DATE OF OBSERVATION	
				Third	Seventh
70528	36	M	M. P.	++++	+++
73766	30	F	A. R.	++	++
75356	34	M	F. R.	+++	+++
75401	28	M	F. P.	++	++
72295	30	F	I. G.	++	++
72879	16	M	A. A.	++	++
72554	16	F	E. D.	++	+
73279	24	F	A. T.	++	+
65949	45	M	M. B.	+	+
70816	11	M	V. C.	+	+
62629	47	M	W. R.	+	+
69568	53	M	L. S.	0	0
71086	48	M	E. C.	0	0
70830	45	M	I. W.	0	0
66106	29	F	S. R.	0	0
73579	43	M	W. K.	0	0
68120	48	M	C. B.	0	0
73586	50	M	J. K.	0	0
73416	42	F	A. W.	0	0
74938	7	M	R. F.	0	0
75357	57	M	F. D.	0	+
75399	29	F	D. L.	0	+
75399a	36	F	J. C.	0	+
68809	67	M	J. S.	0	0

removal.   The alcoholic extract had been used in the tests and in impregnating the bits of blotter with the extract, an excess was avoided, because such an excess would have prevented the proper adhesion of the patch.   These cases, therefore, suggest the possibility that the typical dermatitis of poison ivy develops not only at the point of original contact with the active principle

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but also at times upon skin surfaces distant from the primary lesion, by transference of the active substance through the blood and lymph. While this explanation appears plausible, the possibility must be considered that some of the active substance diffused through the adhesive material, in which it is readily soluble, and thus arrived, either at the edges of the patch, or on the external surface of the canvas, from which sites it could be transferred to other parts of the body by external routes.

A correlation of the different degrees of experimental susceptibility noted above, with the degree of clinical susceptibility in the

TABLE 4
Tests upon group of boys

CASE NUMBER	AGE	CLINICAL HISTORY	CLINICAL ONSET	DAY OF OBSERVATION		
				Third	Seventh	Fourteenth
109	14	General, face and body	2	++++	++++	++
112	15	General, face and body	2	+	+	0
117	20	Severe on hand and feet	2	+	+	0
114	16	Severe on hands	2	+++	+++	+
115	17	Severe on feet	2	++++	++++	++
116	18	Slight	2	++	++	0
110	15	Slight	2	++	++	0
111	15	Slight	3	+	+	0
108	14	Slight	2	++	+	0
106	13	Slight, on extremities	3	+	+	0
105	13	Negative	0	+	+	0
107	14	Negative	0	0	0	0
113	15	Negative	0	0	0	0

same individuals, could not be undertaken in these series since most of the individuals were of foreign birth, and were not acquainted with poison ivy or its natural effects. However the opportunity for making such a correlation was offered in a group of boys. The protocol of these comparative observations is presented in table 4. The group consisted of thirteen boys between thirteen and twenty years of age, who, in midsummer, pitched camp at a site covered with poison ivy vine. Ten of the thirteen gave the clinical history of ivy poisoning as the result of this experience. The tests, in two of the three clini-

cally negative cases, were negative. The third clinically nega-
tive case gave a faintly positive reaction (one plus) by patch
test on the third day, but this reaction was as pronounced as in
four other cases clinically positive, in two of whom the attack
was mild, in two, severe. All of the cases giving severe test
reactions had suffered severe poisoning while in camp. Two
other cases that had been severely affected while in camp gave
only faintly positive reactions. No individual that had exhibited
any degree of clinical susceptibility was negative on test. The
clinical incubation period of all the cases was practically the same,
the dermatitis appearing upon the evening of the second day in
all but two of the cases. In these two cases the lesions occurred
upon the morning of the third day.

In general it can be said that, with the kind of extract employed,
there is a degree of parallelism between the results of the tests
and the clinical history of the cases.

AGE INCIDENCE

Of the eighty individuals included in group 1, 52, or 65 per
cent, responded with a positive reaction to the patch test. All
but one of these individuals were eight years of age, or over.
The number of tested individuals in the different age periods is
too small to permit a definite conclusion as to a possible differ-
ence in susceptibility in poison ivy among individuals over eight
years of age. For purposes of comparison however, we may take
65 per cent as representing approximately the average suscepti-
bility of the individuals over eight years of age with the technic,
and with the use of the alcohol or chloroform extracts described
above. Through the courtesy of Dr. F. H. Bartlett oppor-
tunity was afforded to apply the patch tests in 18 infants be-
tween five weeks and eighteen months of age, all patients in
Dr. Bartlett's service at the Babies' Hospital, New York City.
The results of these tests are shown in table 5. Two tests were
carried out in each case. On one arm the alcoholic extract was
applied by means of the patch test. On the other arm the residue
of the chloroform extract was employed in the manner previously
described. It is seen that no positive reaction occurred by either
test.

TABLE 5

Tests upon infants

CASE NUMBER	AGE	SEX	DAYS OF OBSERVATION		
			Third	Seventh	Tenth
	months				
118	1¼	F	0	0	Home
119	2	F	0	0	0
120	4½	M	0	0	0
121	5	M	0	0	Home
122	5	M	0	0	0
123	6	M	0	0	0
124	6	F	0	0	0
125	6½	M	0	0	Home
126	7	F	0	0	0
127	7	F	0	0	0
128	8	F	0	0	0
129	8	M	0	0	Home
130	10½	M	0	0	0
131	11	M	0	0	0
132	11	F	0	0	0
133	12	F	0	0	0
134	12	F	0	0	Home
135	16	F	0	0	0
136	18	M	0	0	0

SUMMARY

1. The typical vesicular lesion of *Dermatitis venenata* can be produced by means of an alcoholic or chloroform extract of the fresh leaves of *Toxicodendron radicans*, applied to the skin surface.

2. In our hands, the typical vesicular lesion of *Dermatitis venenata* could not be produced by the intradermal injection of an active alcoholic extract. The lesion thus produced was not different from that caused by the intradermal injection of the solvent.

3. With the patch test, differences can be demonstrated in the susceptibility of different individuals to poison ivy, and in the incubation period of the lesion.

4. With the technic used, infants could not be shown to be susceptible.

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

VII. THE AGE INCIDENCE OF SERUM DISEASE AND OF DERMA-
TITIS VENENATA AS COMPARED WITH THAT OF THE
NATURAL ALLERGIES

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Any classification of the various forms of hypersensitiveness
must be considered tentative so long as the mechanism of these
phenomenona remains unknown. In the absence of this know-
ledge, however, it is useful to group the phenomena according
to the facts at hand. Thus, anaphylactic hypersensitiveness can
be properly separated from all the other forms because there is
no satisfying evidence that these latter are dependent upon an
antibody-antigen mechanism, as is the former.

In a previous publication the author associated the drug, food
and animal idiosyncrasies, hayfever, asthma, serum disease and
dermatitis venenata under the term allergy. Two of these
conditions are distinguished from the others by noteworthy
peculiarities. Serum disease presents the almost constant
characteristic of the incubation period and dermatitis venenata
differs from the others in which the skin is involved, in the con-
stant and characteristic nature of the lesion. It has been re-
cently found, also, that serum disease differs strikingly from most
of the other forms of human hypersensitiveness in its percentage
incidence. The observations of Longcope and Mackenzie and
of Rufus Cole supported by statistics collected in the Boston
City Hospital (1) indicate that about 90 per cent of the white
race are susceptible to ordinary serum disease upon *intravenous*
administration of large quantities of horse serum. A high per-
centage of susceptibility also to poison ivy is indicated by some

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experiments by Cooke and by the writer; which will be presently
described. According to Cooke and Vander Veer (2), on the
other hand, only about 10 per cent of the white race are subject
to the natural allergies.[1]

These differences are all so wide that they suggest a difference
in the underlying mechanism of the respective phenomena,
notwithstanding the apparent association of all of them as re-
vealed in the general lack of susceptibility in the American
Indian (1).

It is the purpose of the present communication to describe an
additional difference between the natural allergies and the two
other forms of human hypersensitiveness as well as a certain
apparent difference between these latter two.

In their notable paper on the "human sensitizations," Cooke
and Vander Veer (2) showed that the age incidence of hayfever,
asthma and the food, drug and animal idiosyncrasies is governed
by heredity. For example, if both parents are affected, 36.3 per
cent of the potentially susceptible offspring begin to exhibit
symptoms within the first five years of life. This figure is in
significant contrast with the percentage incidence for this period
of life among potentially susceptible individuals whose antecedent
family history is *negative* with respect to natural allergies; that
incidence is only 5.1 per cent.

From table 2 of the paper by Cooke and Vander Veer can be
calculated the age incidence of all potentially susceptible in-
dividuals without regard to the family history This incidenced
taken in successive five year periods is 11.6, 16.2, 15.2, 11.6,
13.8. 11, 9.8, 6, 1.2 per cent, with 3.6 per cent for the entire
period over 45 years. With these figures we can see how many
of one generation of potential susceptibles have begun to exhibit
symptoms in the different age periods by taking the sum of the

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[1] In this paper the expression "natural allergies" will be used for the sake of
convenience to designate those forms of human hypersensitiveness which result
from natural contact with the exciting agent. For the writer's convenience, also,
although quite illogically, the susceptibility to poison ivy will be excluded from
this group, which will comprise those characterized by the symptoms of hay-
fever, asthma, multiform eruption, angio-neurotic edema and some other symp-
toms after natural contact.

individual percentage for all of the preceding age periods. Thus,
11.6 per cent of the potential susceptibles present symptoms by
by the fifth year; 27.8 per cent present symptoms by the tenth
year; 43 per cent are affected by the fifteenth year and so on up
to the last age period of over 45 years when all of the potential
susceptibles or 100 per cent have become affected.

Thus, it is evident that as a generation advances in years the
number of its members affected with the natural allergies con-
stantly increases.

The percentages taken from table 2 of Cooke and Vander Veer
are calculated with reference to the number of potentially sus-
ceptible individuals and not to the whole number of individuals
in the generation. However, it is evident that if the correspond-
ing percentages could be calculated with reference to the entire
generation these would be merely much smaller than those re-
ferring to the susceptible group; their mathematical ratios to
one another would remain unchanged.

In other words, the percentage of individuals affected with the
symptoms of the natural allergies increases considerably in each
generation, at least in the early successive life periods. Thus
the percentages in the second and third life periods (27.8 and 43)
are respectively about $2\frac{1}{2}$ and 4 times as great as that of the first
life period (11.6).

With these facts as a basis of comparison, a study of the age
incidence of serum disease and of dermatitis venenata (poison
ivy) was undertaken.

The data used in the study of serum disease were obtained in
the Durand Hospital of the John McCormick Institute for
Infectious Diseases in Chicago (service of Dr. G. H. Weaver)
and in the Municipal Hospital in Philadelphia. We are in-
debted to Dr. L. Hektoen for permission to use the Durand
Hospital records and to Miss Adeline S. Lorge for collecting the
data in that hospital. Acknowledgment is due, also, to Mr.
Joseph Peacock and to Miss Helen I. Barrett, who collected the
Philadelphia data.

In table 1 the cases in the two institutions are arranged in five
divisions according to age. In the first column of each division

is given the total number of individuals receiving injections of serum; in the second column is the number of those in whom serum disease was noted; in the third column is the percentage incidence of serum disease.

Differences in the percentage incidence in the different life periods are indicated in the two series of cases. However, it seems significant that those differences are much greater in the Chicago series than they are in the relatively much larger Philadelphia series and that when the two series are combined the

TABLE 1

Showing the age incidence of serum disease

	0-5 YEARS			6-10 YEARS			11-15 YEARS			16-25 YEARS			25 YEARS UP		
	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease
Chicago...	425	75	17.9	314	65	20.7	136	17	12.5	190	42	22.1	142	21	14
Philadelphia	2215	195	8.8	1498	161	10.8	331	37	11.1	376	36	9.6	233	18	7.7
Total ..	2640	270	10.2	1812	226	12.5	467	54	11.6	566	78	13.8	375	39	10.4

differences are reduced to relative insignificance. It seems probable, in other words, that as the number of individuals under observation in the different age groups increases, the differences in the percentage incidence will tend to diminish.

It must be freely admitted that the clinical records of the incidence of serum disease are practically never perfect. However, it seems most unlikely that this inaccuracy could be much greater for one age period than for another. Hence, the conclusion may be confidently drawn from the foregoing statistical study of serum disease that the incidence of this condition is not materially different in the different age periods.

The determination of the age incidence of sensitiveness to poison ivy is attended with the difficulty that many individuals either have not come in contact with this plant or have not recognized the nature of the lesion which resulted from such contact. Trustworthy data in these circumstances could be obtained only by experiment. This was carried out by applying an extract of the leaves of poison ivy to a small area of the skin of a number of individuals and observing the effect after 48 hours contact. The extract was made by grinding the young leaves in a meat-chopper and mixing the resulting moist mass either with 95 per cent alcohol or with chloroform.

Both of the extracts were filtered through paper after three days contact with the ground leaves. The alcoholic extract was used in the crude fluid form. The chloroform extract was placed in a broad shallow dish before an electric fan until all of the chloroform had been evaporated and only a sticky residue remained.

These extracts were applied to the skin according to a method employed by Robert A. Cooke in some unpublished experiments and referred to by him as the "patch" method or test. The fluid alcoholic extract was applied to the skin by soaking a small square of blotting paper with it and keeping the impregnated paper in contact with the skin with the use of adhesive-plaster.

The residue of the chloroform extract was smeared on a small area of the skin over the anterior surface of the forearm and the area was protected with the use of adhesive plaster. Care was taken to prevent contact of the adhesive preparation on the plaster with the ivy extract as the latter is very soluble in the adhesive material and is quickly taken up into it from the skin surface.

Observation of the individuals so treated was continued until a lesion appeared or at least for five days. The test was carried out upon twelve adults over 20 years of age and of these only 1 remained unaffected. The lesions that developed in the eleven susceptible individuals resembled those of dermatitis venenata in their vesicular nature and in the itching; they remained local.

The high percentage of susceptibility among these adults is in agreement with that noted by Cooke in the unpublished experiments referred to above.

As soon as these results were obtained the test was applied in a series of twelve children five years of age or younger. Two of these were under one year; the others ranged between three and five years. Only one of these was found susceptible to the ivy extract—a boy of five years. These children were patients in the New York Hospital in the service of Dr. J. C. Roper, who kindly permitted the tests to be carried out and who controlled the results.

At this point the investigation was interrupted but it was thought that the results of even these two short preliminary series give sufficient indication of a great difference in susceptibility to poison ivy between young children and adults. This indication is rendered a practical certainty by the findings of Dr. Spain (3).

From these experiments it seems that the age incidence of poison ivy susceptibility differs from that of both the "natural" allergies and serum disease.

The susceptibility to serum disease is exhibited by a constant proportion of individuals in all of the life periods. That to the "natural" allergies is shown by a proportion of individuals which increases rapidly in the early five-year periods. On the other hand, while the susceptibility to poison ivy is exhibited by a proportion of individuals which increases from birth to adult life, like that to the "natural" allergies, it differs strikingly from the latter in the very high percentage susceptibility of the adults—about 90 per cent as contrasted with the 10 per cent general incidence found by Cooke and Vander Veer (2). This difference is really increased by the fact that the 10 per cent incidence of the natural allergies found by these authors includes susceptibility to a number of substances; it cannot, therefore, be fairly compared with the incidence of susceptibility to the single substance—poison ivy.

It is not possible to interpret the differences that have been found among the different forms of human hypersensitiveness

referred to.   While those differences appear to indicate a different origin or mechanism of the three forms, judgment in this question must await further knowledge.

SUMMARY

1. Serum disease differs from other forms of human hypersensitiveness in the almost constant characteristic of the incubation period and in its high percentage incidence.

2. Dermatitis venenata differs from the other forms of human hypersensitiveness in which the skin is affected in the constant and characteristic nature of the lesion.

3. Statistical study and some experiments reveal the following differences in the age incidence of the "natural" allergies, serum disease and dermatitis venenata:

a. The age incidence of the "natural" allergies increases rapidly in the early age periods but probably does not greatly exceed 10 per cent in any period.

b. The age incidence of dermatitis venenata increases greatly from childhood to adult life and reaches a high percentage (probably about 90 per cent).

c. The age incidence of serum disease seems not to change during life.

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

VIII. ON THE RELATIVE SUSCEPTIBILITY OF THE AMERICAN INDIAN RACE AND THE WHITE RACE TO THE ALLERGIES[1] AND TO SERUM DISEASE

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The fact that serum disease generally appears after an incubation period following the injection of an antigenic protein and is usually accompanied by the formation of specific antibody (precipitin) would seem to separate this form of human hypersensitiveness from those which are exhibited immediately upon natural contact with the exciting agent in the entire absence of demonstrable antibodies. The former condition seems to be experimentally induced and dependent merely on a suitable previous contact with the exciting agent—serum protein: the latter, according to the studies of Cooke and Vander Veer, appear to be wholly subject to an hereditary factor and not dependent upon previous contact with the exciting agent.

Our knowledge of these conditions is drawn from observations upon the white race. Certain publications, to which we shall refer again, seemed to indicate that some of the forms of hypersensitiveness in human beings (asthma, dermatitis venanata) are rare among the American Indians and it was thought that investigation of this apparent racial difference might produce some information regarding the nature of the different forms of specific human

[1] By allergy is meant the inherited forms of specific human hypersensitiveness, such as hay-fever, asthma, angio-neurotic edema and urticaria.

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hypersensitiveness as well as some indication of the relation of the American Indians to the other human races.

Particularly, it seemed desirable to find out first, whether the American Indian is relatively infrequently affected by the inherited forms of hypersensitiveness such as asthma and hay-fever and secondly, whether or not in such case the induced condition of serum disease is also infrequent in that race.

If the incidence of serum disease were found to be equal in the Indian race and in the white race, this form of human hypersensitiveness would be, thereby, the more sharply differentiated from the inherited form. If, on the other hand, both forms of hypersensitiveness were found to be equally infrequent, then an identity in at least a part of the underlying mechanism of the two forms would be indicated.

Through correspondence with physicians that had had extensive medical experience among American Indians we learned that hay-fever, asthma, urticaria and drug allergy are very uncommon in the full blooded individuals of that race. We are able, with the coöperation of Dr. N. P. Sherwood, Professor of Bacteriology in the University of Kansas, and Mr. H. B. Pearis, Superintendent of Haskell Institute in Lawrence, Kansas, to observe the occurrence of serum disease in twenty-six students in Haskell Institute, all listed as full blooded American Indians.

THE OCCURRENCE OF THE ALLERGIES AMONG AMERICAN INDIANS

A search of the literature has revealed but few references to the incidence of allergic conditions in the Indians.[2] In his report on Physiological and Medical Observations among the Indians of Southwestern United States and Northern Mexico (1), Aleš Hrdlička remarks on page 188, "No instances of severe asthma were encountered," and on page 191, asthma is "rare among Southwestern Indians."

In his article on "Skin Diseases among Full Blooded Indians of Oklahoma" (2), E. S. Lain writes:

[2] In this article the word Indian always refers to the American Indian.

The extreme rarity of dermatitis medicamentosa, dermatitis venenata, may partially be accounted for by the lack of common usage of drugs, soaps, and other chemicals, though the Indians frequently apply quite freely on their faces and other exposed parts paints and dyes made from the wild plants, berries, etc. The skin of the Indian is apparently almost immune to the poison ivy and other plants which cause so much discomfort to the women of our race.

Our own inquiry was made in the form of a questionnaire, answers to which were received from eleven physicians and one superintendent of an Indian school. The replies were based on extended medical observation of about 40,000 full blooded Indians.

Those replying to the questionnaire were:

Dr. A. J. Anderson, Lawrence, Kansas.

Mr. Clyde M. Blair, Superintendent Chilocco Indian School, Chilocco, Oklahoma.

Dr. A. D. Lake, Gowanda, New York.

Dr. Otis E. Lovelady, Ponca Agency, Whiteagle, Oklahoma.

Dr. A. E. Marden, W. S. Indian Vocational School, Phoenix, Arizona.

Dr. James M. Meason, Pima School, Sacaton, Arizona.

Dr. Charles M. Ming, Okmulgee, Oklahoma.

Dr. W. W. Rublee, Sherman Institute, Riverside, California.

Dr. F. A. Spafford, Flandreau, South Dakota.

Dr. A. J. Wheeler, W. S. Indian Vocational School, Phoenix, Arizona.

Dr. Lawrence White, Keshena, Wisconsin.

Dr. A. M. Wigglesworth, Albuquerque, New Mexico.

The essential questions proposed were:

1. Have you observed the occurrence of asthma or hay-fever in full-blood Indians?

2. Do these conditions occur in the white population of the vicinities in which the observations upon the Indians were made?

3. Have you observed the occurrence of urticaria in full-blood Indians?

In all of the replies question 2 was answered in the affirmative.

The following men could recall no instance of asthma, hay-fever or urticaria in a full-blood Indian: Mr. Blair, Drs. Lovelady, Meason, Anderson, E. F. Menger, Ming, Roblee, Spafford, Wheeler, and White.

Dr. Marden has seen "two or three cases of asthma," and "numerous cases of urticaria" in full-blood Indians (about 10,000 individuals under observation).

Dr. Menger has seen "hay-fever, asthma, urticaria and dermatitis venenata *only* in the breeds" (about 1500 individuals under observation).

Dr. Wigglesworth has seen hay-fever and asthma in full-blood Indians and "urticaria many times." (About 15,000 individuals under observation.)

Dr. Lake has seen "a few cases of hay-fever and of asthma." He states that "hives are common and poisoning from ivy is frequent." However, he writes that "very few" of the Indians under his observation are full-blooded.

These replies suggest that while the allergic trait is not absent in the Indian race this character is very much less marked in the Indian than it is in the white race.[3] The observation of allergic symptoms in some pure Indians makes it inadmissible to assume the complete absence of the basic condition in all of the Indians that do not naturally present the outward signs of it.

It may be that, exactly as in the white races, some individuals are hypersensitive in a degree too slight to allow the development of symptoms upon the *natural contact* with the exciting agent. In such individuals the *injection* of the agent would reveal the latent condition.

From the foregoing facts it could have been anticipated that if serum disease is associated in some way with the allergies it should occur in full-blood Indians upon injection of serum, but much less frequently and possibly in milder form than it does in the white race. In fact, this was the outcome of the experiences which we are about to describe.

[3] In the same inquiry the statement of Lain regarding the relative insusceptibility of the Indians to "poison ivy" was confirmed.

We had learned from Dr. W. T. Longcope that in his experience symptoms of serum disease occur in at least 90 per cent of white individuals receiving 100 cc. or more of whole horse serum by intravenous injection.

With this high percentage as a standard of comparison it was thought that at least some indication of the relative susceptibility of the two races to serum disease could be obtained from the results of the intravenous injection of about 100 cc. of normal horse serum into even a few of the Indians.

The observations were made upon twenty-six full-blooded Indians, all students in Haskell Institute situated in Lawrence, Kansas.   We consider it a privilege to record the fearlessness and patience with which these intelligent young men, most of whom were fully aware of the investigatory nature of the procedure, submitted to the injections.   It is not possible to distinguish any individuals among them in the respect of courage or fortitude. Those who presented themselves in the first series faced uncertainty as to the possible pain and danger attending the injections. Those that came later came with the knowledge of the unpleasant symptoms which some of their predecessors had suffered.   It should be recorded that the two who were the last to be injected had witnessed the effect of the injection upon Charlie Hutchison. These two were given ample opportunity to withdraw, but neither would take advantage of that suggestion.

THE OCCURRENCE OF SERUM DISEASE IN THE AMERICAN INDIAN

The material injected was fresh normal horse serum preserved with the addition of 0.6 per cent of a mixture of equal parts of "Three Cresols" and ether.   According to Krumwiede and Banzhaf (3), one of the unpleasant immediate effects (chills) of the injection of serum preserved with cresol is avoided by adding the preservative mixed with an equal volume of ether.   It may be stated here that this symptom was absent in all but one of the individuals in the present series (Jesse King).

The serum was prepared especially for this investigation in the laboratories of the H. K. Mulford Company and delivered

to us in double stoppered 110 cc. tubes together with a number of the H. K. Mulford Company intravenous outfits.[4] With these convenient outfits we were able to make the injections at the rate of six in an hour.[5]

Previous to the injections cutaneous tests were made with dried horse serum prepared by the H. K. Mulford Company. All of these resulted negatively.

The serum was brought to body temperature by placing the tubes in warm water.

The effects of the injections in the different individuals were as follows:

1. William Frank (Creek). Received 110 cc. Slight headache on the day of injection and temperature of 99.8 F. Presented no other symptoms at any time.[6]

2. Joe Bearhead (Cheyenne). Received 110 cc. Complained of headache on day of injection. Had some epistaxis on the third day. On the 10th day there were a few urticarial spots on the arms and body without elevation of temperature; these were present also on the 11th day still without fever, but they had disappeared by the 12th day.

3. Benjamin Osage (Cheyenne). Received 110 cc. Immediately after the injection a swelling 1 cm. in diameter appeared on the left forearm just above the wrist. The swelling was situated more deeply than the usual urticarial wheal. A smaller spot appeared on the forehead and another 2 mm. in diameter above the one on the forearm. There was severe headache on the day of the injection with a temperature of 100°F. There were no further symptoms.

4. Harber Johnson (Creek). Received 110 cc. There was immediate injection of the conjunctiva with some swelling of the eyelids and face. There was headache on the day of the injections, but no symptoms thereafter.

5. William Atkins (Otoe). Received 25 cc. There were no symptoms at any time.

[4] We are under great obligation to Drs. John Reichel, F. M. Huntoon and J. A. Murphy of the H. K. Mulford Company for the care with which the serum was prepared and delivered to us.

[5] Acknowledgement must be made of the skillful assistance of the nurses— all Indian girls—under the direction of Miss Anderson, superintendent of nurses in the Institute.

[6] There was some coughing during the injection or immediately afterward in most instances and in some a slight sense of oppression in the chest.

6. Thomas Wasson (Baiut). Received 110 cc. Within ten minutes after the injection two urticarial spots appeared on the eyelid and the neck. There were no further symptoms.

7. Delmar Scott (Mojave). Received 110 cc. There were no symptoms until the 8th day when there was fainting and a temperature of 100°F. On the 9th day generalized urticaria appeared which lasted three days. There was no elevation of temperature on the 9th day nor thereafter.

8. Robert Leve Leve (Walapi). Received 110 cc. There were no symptoms until the 12th day when there were severe joint and muscle pains without temperature elevation or eruption.

9. William Hampton (Choctaw). Received 110 cc. Complained of joint pains on the 5th day and again on the 10th day when some urticarial spots appeared. The joint pains continued on the 11th and 12th days but there was no urticaria after the 10th day. There was no elevation of temperature at any time.

10. Lawton Raymond (Navajo). Received 110 cc. There were no symptoms at any time.

11. Joseph Parnell (Assiniboin). Received 110 cc. There were no symptoms at any time.

12. Wallace Littlefinger (Sioux). Received 110 cc. On the 11th and 12th days there was urticaria without fever and on the 13th day there was a temperature of 99°F. with joint pains but without urticaria.

13. Edward Davenport (Sac and Fox). Received 110 cc. There were no symptoms at any time.

14. Emery Redbird (Ottawa) Received 110 cc. There were no symptoms at any time.

15. Morris Baken (Choctaw). Received 20 cc. On the 11th day there was swelling of the left parotid gland without fever. On the 12th day there were joint pains with a temperature of 100°F. No symptoms thereafter.

16. William Ruskin (Navajo). On account of the very small size of the vein a double puncture occurred causing a hematoma, which prevented the injection.

17. Marion Runsthru (Assiniboin). Received 110 cc. There were no symptoms at any time.

18. Andy Snap (Creek). Received 110 cc. On the 6th day there was urticaria without fever; on the 7th day the urticaria had disappeared but there were cramps and weakness with a temperature of 99°F. Urticaria reappeared on the 8th day accompanied by edema of the face and

a temperature of 100°F.   There were no symptoms on the 9th day nor thereafter.

19. Jesse King (Creek). Received 95 cc. There was immediate marked uneasiness with pain in the chest and about the eyes. Pulse at eight minutes 140; at thirteen minutes 118, and at eighteen minutes 104. At thirty minutes he was able to walk out. At one hour there was a chill. There were no further symptoms.

20. Mason Kawaykla (Apache). Received 110 cc. There was urticaria on the 10th and 11th days without fever or other symptom.

21. Walter Emarthla (Creek) Received 110 cc. There were no symptoms at any time.

22. James Foster (Creek). Received 110 cc. There were a few urticarial spots on the 10th day without fever or other symptoms.

23. Abel Archibald (Creek). Received 110 cc. There were no symptoms at any time.

24. John Alonzo (Pueblo). Received 90 cc. Vomited immediately after the injection, but presented no further symptoms at any time.

25. Charles Hutchinson (Arapahoe). Received 80 cc. Immediately complained of pain in the chest. There was edema of the eyelids and congestion of the conjunctivae; a general weakness and a rapid and weak pulse (116) five minutes after the injection. Had to be carried to bed There was severe vomiting after two hours. The edema of the eyelids and the weakened heart action persisted into the following day. Thereafter there were no further symptoms. There was a previous history, in this case, of a weak heart and a constitutional weakness.

26. Andrew Juan (Pima). Received 110 cc. There was headache and a temperature of 100°F. on the 2nd day, but no further symptoms at any time thereafter.

27. Edward Meeks (Arapahoe). Received 110 cc. There was a slight malaise immediately after the injection and on the 9th day urticaria without elevation of temperature.

In considering what symptoms were to be regarded as indicative of "serum disease" we have been guided, naturally, by the attitude of those upon whom we depended for our "control" series among the white race, that is Dr. W. T. Longcope and Dr. Rufus Cole. These observers have used only eruption and joint pains with or without fever as criteria of that condition, and as is customary they have ignored the immediate effects of the injections, excepting eruption. We have, therefore, omitted

from consideration the effects noted in the cases of William Frank, Harber Johnson, Jesse King, John Alonzo, Charles Hutchison and Andrew Juan.

In table 1 is given a summary of the symptoms that could be regarded as those of "serum disease" resulting from the injections into the Indians.

It is seen that the incidence of the condition among those injected was 46 per cent; the average duration of the symptoms was two days and the average elevation of temperature was 0.38°F.

In comparing the course of serum disease as just described in the Indians with that in the white race it must be pointed out that the injections made in the Indians were given to healthy young men who were under orders to report to the physician in charge (Dr. E. F. Menger) the slightest ailment. For this reason the existence of serum disease was recognized in several of the Indians by symptoms (such as joint pains and edema and by the immediate appearance of a few urticarial spots) which are rarely noted in the usual medical history. It was necessary, therefore, in order to obtain comparable statistics among white individuals to seek them in institutions in which the occurrence of serum disease is given particular attention. These requirements were amply met in the records of a series of cases in the Presbyterian Hospital of New York City which were under the care of Dr. W. T. Longcope and Dr. George M. Mackenzie. We are indebted to these observers for permission to publish the data and to Dr. E. P. Maynard by whom the data were collected.

It is evident that the relative susceptibility of the two races should not be judged solely by the relative percentage incidence. A fair comparison should include also the factors of duration of the disease and its severity. As an index of severity we have used the single feature of elevation of temperature. In a series of fifty-two individuals treated with serum (horse) injections in the Presbyterian Hospital and observed continuously for one month, serum disease occurred in forty-eight instances—an incidence of 92.4 per cent.

TABLE 1

Showing the clinical course of serum disease in full blooded Indians

DAY OF INJECTION

	1st	5th	6th	7th	8th	9th	10th	11th	12th	13th
Baken (Choctaw).........								Swelling of parotid. 98.6°F.	Joint pains. 100°F.	0
Littlefinger (Sioux)......							Hives. 98.6°F.	Hives. 98.6°F.	Hives. 98.6°F.	No hives. Joint pains. 99°F.
Kawaykla (Apache)......								Hives. 98.6°F.	0	
Foster (Creek)...........							Hives (few). 98.6°F.	0		
Hampton (Choctaw).....						0	Joint pains. Few hives. 98.6°F.	Joint pains. No hives. 98.6°F.	Joint pains. No hives. 98.6°F.	
Snap (Creek)..........		Joint pains. 98.6°F.	Hives. 98.6°F.	Cramps. Weakness. No hives. 99°F.	Hives. Edema face. 100°F.					

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Meeks (Arapahoe)........					0
Scott (Mojave)...........	Weak-ness. 100°F.	Hives. 98.6°F.	0	Hives. 98.6°F.	0
Leve Leve (Walapi)......		General hives. 98.6°F.	Few hives. 98.6°F.	Few hives. 98.6°F.	Joint pains. No hives. 98.6°F.
Bearhead (Cheyenne).....					0
Osage (Cheyenne)........	Immediately, few urticarial spots. No further symptoms.				
Wasson (Baiut)..........	Immediately, few urticarial spots. No further symptoms.				

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In table 2 the duration of the condition in forty-two of the cases is given and for direct comparison the duration of the condition in the twelve Indians.   The average duration in the white individuals was four and one-half times as great as it was in the Indians.

As an index of the relative severity of the serum reaction, comparison has been made of the average elevation of temperature above the normal level.

TABLE 2

Duration of serum disease after intravenous injection of serum

IN 42 WHITES (PRESBYTERIAN HOSPITAL) NEW YORK CITY						IN 12 FULL-BLOODED INDIANS (HASKELL INSTITUTE)	
Days	Cases	Days	Cases	Days	Cases	Days	Cases
1	1	9	1	15	2	1	5
3	3	10	1	16	1	2	3
4	11	11	1	20	2	3	1
5	6	12	1	23	1	4	3
6	2	13	1	24	2		
7	4	14	1	25	1		
Average duration 9 days						Average duration 2 days	

In determining the average elevation of temperature in the white individuals only those cases were considered in which the temperature had been normal before the symptoms of serum disease had appeared.   As all of these individuals had been suffering from a bacterial infection they were, according to Dr. Mackenzie, who selected the cases, possibly more prone to temperature elevation than a normal individual.   Dr. Mackenzie suggests, therefore, that this instability be accounted for in estimating the temperature elevation in these individuals by advancing the normal level somewhat.   As the temperatures in these cases, were taken per rectum, Dr. Mackenzie suggests that 100°F. be taken as the normal temperature.   This suggestion has been adopted.

Following are the highest rectal temperatures observed in thirty-seven of the Presbyterian Hospital cases during the period in which the individuals presented symptoms of serum disease:

104.0	101.0	104.8
100.6	99.8	101.6
103.0	99.4	100.0
100.6	103.6	101.2
101.0	103.0	102.2
103.0	99.6	100.0
100.0	100.0	104.0
100.2	102.4	99.6
100.2	100.0	100.0
99.8	100.2	99.2
100.2	99.4	102.0
101.2	100.4	
99.8	102.2	

The total elevation of temperature over 100°F. in all of these individuals was thus 42.6°, or an average elevation of 1.15°F. The total elevation of temperature over 98.6°F. (by mouth) in the twelve affected Indians was 4.6°F., or an average elevation of 0.38°F.

TABLE 3

Comparison of the serum reactions of the white and Indian races

	WHITE	INDIAN
Serum-treated cases followed completely....................	52	26
Cases of serum disease.....................................	48	12
Percentage incidence.......................................	92.4	46
Average duration (days)...................................	9—	2
Average temperature elevation, degrees F.................	1.15	0.38

Ratio in which the two races are affected:

$$\frac{92.4}{46} \times \frac{9}{2} \times \frac{1.15}{0.38} : 1 = 27:1$$

In table 3 is presented a summarized comparison of the occurrence of serum disease in the two races. The ratio in which the two races are affected is properly estimated by multiplying together the single ratios of the three different factors—incidence, duration and severity. By this calculation the susceptibility of the white race is found to be about twenty-seven times as great as that of the Indian.

Objection must be made to the placing of much dependence upon the figures used in this calculation on account of the small-

ness of the number of individuals in both of the series that form the basis of comparison. In the circumstances, access cannot be had to further data as to serum disease in the Indian. However, we are able by the courtesy of Dr. Rufus Cole to supplement the observations in the white individuals with his own experiences in the Hospital of the Rockefeller Institute. It was important to obtain further data upon the white race because of the surprisingly high percentage of incidence observed in the Presbyterian Hospital—the highest, by far, that has yet been reported. We quote here from a personal communication from Dr. Cole:

No intensive study of serum disease has been made at The Hospital of The Rockefeller Institute and any statistical conclusions drawn from our experience cannot be considered final. However, I have collected 223 cases in which amounts of serum varying from 30 cc. to 2000 cc. were administered. Only a few of the patients, however, received the very large amounts; most patients received from 200 cc. to 400 cc. during a period of two to three days. We have records concerning the occurrence of urticaria, skin rash, enlarged glands, red and painful joints, and fever. The statistics regarding fever will have to be studied much more carefully before much stress can be laid upon them, since in many pneumonia patients who have received serum, it is impossible to say whether a late elevation of temperature is the result of the administration of the serum or is due to some complication.

Among the 223 cases, the occurrence of a skin rash, usually urticaria, at some time following the injection of serum, was noted in 158 patients; i.e. in 70 per cent. The rash appeared from one to thirty days following the injection of serum and varied in degree from a few urticarial wheals to urticaria covering almost the entire body. In a number of cases there occurred an erythematous rash; in a few cases it was scarlatiniform or morbilliform. Thirty-eight of the 223 cases ended fatally; in a number of instances, death occurred within a few days after the administration of serum before sufficient time had elapsed for a rash to develop. In only 9 of these 38 cases was the occurrence of a rash noted. If these 38 cases are omitted, 185 cases are left, of which 149 showed a rash at some period; i.e. 80 per cent.

The occurrence of painful joints was noted in a considerably smaller number of cases, only in 68 of the 185 cases, and general glandular enlargement was noted in only 36 of the 185 cases. In some cases, however, enlarged glands, or painful joints, were noted where there was

no skin rash, so that among the 185 cases there occurred 153 in which skin rash, glandular enlargement, or painful joints were present at some period, namely, in 85.4 per cent of the cases.

When our former statements regarding the frequency of occurrence of serum sickness were made, we disregarded those cases in which urticaria occurred immediately following the administration of serum and those cases in which the signs and symptoms were hardly noticeable. Employing, however, your definition of serum disease, at least 85 per cent of our cases may be said to have suffered from this condition, and considering the fact that in a few instances we undoubtedly overlooked very minor manifestations, it is quite probable that the statement that "90 per cent of patients following administration of foreign serum suffer from serum disease" is correct.

Further support of Dr. Cole's opinion was obtained in a study of a series of 367 cases receiving intravenous injections of serum in the Boston City Hospital. We are indebted for permission to publish these statistics to Dr. Edwin H. Place, Physician in Chief to the South Department of the Boston City Hospital. Ninety-nine of this series were cases of pneumonia that had received anti-pneumococcus serum. We are indebted to Miss L. M. Corcoran for assistance in compiling the data on these ninety-nine cases. The average amount of serum injected in these cases was 272 cc. The incidence of serum disease recorded in the histories is 67.7 per cent.

The remaining 268 individuals were cases of diphtheria in the South Department of the hospital and received an average of 150 cc. of diphtheria antitoxin serum. As antitoxin is administered in this department sometimes in the form of the pseudoglobulin fraction and sometimes as whole serum, it was necessary for us to inquire in each instance as to the nature of the preparation used. This was readily done because the laboratory serial number of the preparation injected was always noted in the record. We have been able to obtain this information through the kindness of Dr. Benjamin White, Director of the Division of Biologic Laboratories of the Massachusetts Department of Public Health, in whose laboratories the antitoxin was prepared.

The incidence of serum disease in the diphtheria cases was 70 per cent. It should be noted that the incidence in this series receiving an average of 150 cc. is about the same as that of the pneumonia series receiving 272 cc. Fifty-six individuals received less than 100 cc. and of these fifty-six, eight received less than 50 cc.

These percentages are so close to the original estimated 70 per cent of Dr. Cole that they furnish strong support to his conclusion that some form of serum reaction follows the intravenous injection of horse serum in about 90 per cent of individuals so treated. The correction introduced by Dr. Cole in his original estimate is particularly applicable in the Boston series on account of the fact that all cases that were under observation for ten days or more are included in it.

The evidence presented above seems to leave little doubt as to the approximate correctness of the figure, 92.4 per cent, used in our comparison of the two races to indicate the incidence of serum disease in the white race. This being the case, the estimated ratio of susceptibility of the two races to serum disease may be regarded with some confidence as indicating, at least, a wide difference in the relative susceptibility.

It is seen that a similar difference in the relative susceptibility of the two races is exhibited in both allergy (hay-fever, asthma, urticaria) and serum disease. This fact by no means proves these two conditions to be due to the operation of an identical mechanism. It merely suggests a similarity of mechanism in both conditions, which may not be complete.

SUMMARY

Through inquiry it has been found that the American Indian is apparently much less frequently affected by the allergies than is the white race. An experimental study of the occurrence of serum disease in twenty-six volunteer full-blood American Indians indicates that the Indian race is much less susceptible to that condition than is the white race.

This similarity in the relative susceptibility of the two races to these two conditions suggests a similarity in the underlying mechanism of both of the conditions which, however, need not amount to a complete identity.

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FRONT ROW

WILLIAM   JOE   BENJAMIN   HARBER   WILLIAM   THOMAS   DELMAR   ROBERT   WILLIAM   LAWTON   JOSEPH
FRANK   BEARHEAD   OSAGE   JOHNSON   ATKINS   WASSON   SCOTT   LEVE LEVE   HAMPTON   RAYMOND   PARNELL

MIDDLE ROW

FRANCIS   AGNES   HAZEL   LENNA   NANCY   IDA   MISS   DR.   CHARLES   ANDREW   EDWARD
CORNELIUS HILDEBRAND   DUPUIS   DANN   MCKNIGHT   PRIMEAUX   HANNAH   EDWARD F. HUTCHISON   JUAN   MEEKS
                                                        ANDERSON   MENGER

BACK ROW

WALLACE   EDWARD   EMERY   MORRIS   WILLIAM   MARION   ANDY   JESSE   MASON   WALTER   JAMES   ABEL
LITTLEFINGER DAVENPORT   REDBIRD   BAKER   RUSKIN   RUNSTHRU   SNAP   KING   KAWAYKLA   EMARTHLA   FOSTER   ARCHIBALD

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STUDIES IN SPECIFIC HYPERSENSITIVENESS

IX. ON THE PHENOMENON OF HYPOSENSITIZATION (THE CLINICALLY LESSENED SENSITIVENESS OF ALLERGY)

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In 1911, the writer began the application of the principle of desensitization to the treatment of the allergies (hay fever, asthma, urticaria, angio-neurotic edema).

This principle was employed under the influence of the view of Wolff-Eisner and of Meltzer that the hypersensitiveness of human beings is an expression of anaphylaxis; that is, dependent upon the presence in the sensitive tissue of specific antibodies. Coca (1, 2), in a recent analysis of the phenomena of anaphylaxis and those of allergy, has pointed out differences between these two conditions of such a nature as to make an identity of the two seem unlikely.

One of these differences is a fact that had been observed by the writer in innumerable instances; namely, that although a certain degree of lessened sensitiveness can be obtained in allergy by administration of the exciting agent, either in its natural state by mouth or in extracts by injection, this effect in human beings has never been observed to approach the entire insensitiveness of the condition of "complete desensitization" in anaphylaxis in the lower animals.

In illustration of this principle, the following concrete observations were drawn by Coca out of our unpublished records. In all of the individuals suffering from any form of allergy, who have been rendered clinically insensitive to the natural contact with the exciting agent, the suitable administration of the agent,

by intracutaneous or subcutaneous injection will demonstrate the persistence of the hypersensitiveness.

In some of our cases, indeed, it could be shown that even the insensitiveness to the natural contact was only relative. For example patient A. K., No. 1107,[1] sensitive to egg, was brought by appropriate injections and oral administration of increasing doses of egg white to the point where one whole egg every other day was tolerated, but on several trials he was found unable to eat two eggs on one day without exhibiting symptoms.

The phenomenon of desensitization is a constant characteristic of the condition of anaphylactic hypersensitiveness in all the lower animals in which the phenomenon has been studied. This must be true because the hypersensitiveness of anaphylaxis depends upon the presence of antiprotein antibodies (precipitins) in the tissues; and because it is always possible by a suitable manner of injection of the antigen to neutralize these precipitins so gradually as to avoid the physiological reaction of anaphylaxis and to neutralize them so completely that the hypersensitiveness is *entirely* removed.

It would seem, therefore, that desensitization, which is a constant characteristic of anaphylaxis, should be demonstrable in that condition in every animal in which anaphylaxis occurs. A conceivable exception might exist in an animal in which the antibody production was so rapid that the neutralized precipitins were immediately replaced. However, no such exception is known.

It would seem natural for one seeking to demonstrate desensitization in allergy to choose serum allergy as the first object of study; particularly, the ordinary form of this condition in which the clinical symptom of urticaria is only exhibited after an incubation period following the injection of serum and which is generally accompanied by precipitin formation. However, no one has yet applied the procedure of desensitization to individuals under these circumstances.

In the rarer form of serum allergy, in which symptoms appear

[1] This case is described in greater detail in the article on constitutional reactions; this Journal volume 7, p. 141.

immediately after a primary injection and in which the previous existence of antibodies has not been demonstrated (3), attempts have been made to reduce the sensitiveness by the injection of increasing quantities of serum, beginning with small doses.

In his paper on "Asthma Complicating the Serum Treatment of Pneumonia" (4), H. L. Alexander describes three cases in two of which the procedure just referred to was employed. In one of these the subcutaneous injection of 1 cc. caused urticaria, and the same quantity injected intravenously two and one-half hours later caused nausea, vomiting and asthma. One and a half hours after this occurrence 1 cc. was again injected and this again caused nausea and vomiting, but without an asthmatic attack. Two further injections of 2 cc. and 4 cc. produced no symptoms and a final intravenous injection of 65 cc. was accomplished with no more serious effect than an attack of "hay fever" and of asthma.

Another individual was brought gradually to the intravenous injection of 1 cc. of serum without symptoms. Then the intravenous injection of 40 cc. of the serum produced asthma and subsequent injections of 40 cc., 60 cc., 65 cc., 70 cc., and 75 cc. at from six to eight and a half hour intervals all caused mild or moderate "reactions." Alexander adopted the view, at that time unopposed, that he was dealing with a state of anaphylactic sensitiveness. He overlooked the difficulty attaching to this view by reason of the uniform absence of demonstrable antibodies in the form of serum sensitiveness that he was studying.

In a paper entitled "Serum Desensitization" (5), George M. Mackenzie added two further cases to those of Alexander. In one of these 1 cc. of serum injected intravenously produced respiratory difficulty and marked urticaria. An identical injection twelve hours later caused no symptoms, but after a further interval of one and a half hours, 2 cc. again produced marked urticaria, this time with edema. In the second case 16 cc. of serum produced eruption, although 8 cc. administered three quarters of an hour previously had caused no symptoms.

In all of these experiments the writer's experiences referred to above have been duplicated. A lessened sensitiveness was

attained in a practically important degree, but not complete *insensitiveness*. Moreover, this result was obtained under circumstances—the *repeated intravenous injection of relatively large quantities of serum*—which warrant the conclusion that complete specific insensitiveness is not attainable in allergy.

The uniform failure to induce complete insensitiveness in allergy indicates, according to Coca, that the relative insensitiveness attained in allergic conditions is of a nature quite different from that of desensitization. The objection may be raised against Coca's conclusion that the difference between these two effects need not be a qualitative one; it may be only quantitative. That is, the clinical insensitiveness attainable in allergy may represent a partial desensitization. In fact, a superficial examination favors this latter view.

However, facts are at hand to prove that the difference between the two effects is not quantitative but qualitative. They indicate, by the same token, that the allergic hypersensitiveness of human beings is not due to the influence of precipitin—it is not anaphylaxis.

We are referring to the results published by Coca and Kosakai (6) in their study of the reactivity of partially neutralized precipitin. These investigations showed that the partial neutralization of precipitin takes place according to a law which is quite different from that governing partial chemical precipitation. As they did not analyze their experiments from this point of view, we will do it here.

In their table (1) it appears that in guinea-pigs passively sensitized with 0.4 cc. of a precipitating serum, only slight symptoms were caused by the injection of 0.0025 cc. of the antigen solution. After a partial neutralization of the precipitating serum with a quantity of the antigen corresponding with 0.0008 cc. animals were sensitized with 0.4 cc. of the treated serum and it was found that in these animals 0.01 cc. of the antigen was required to produce slight symptoms; that is, twelve and a half times as much as was used for the partial neutralization.

In a similar experiment (table 4) in which the partial neutralization was carried out *in vivo* with 0.008 cc. of the antigen 0.1 cc. was required to cause symptoms in the sensitized guinea-

pigs—again twelve and a half times the quantity of antigen used for the partial neutralization of the antiserum (partial desensitization in this case).

A third experiment (table 8) with a different serum, resulted in a larger ratio between the dose of antigen used for partial desensitization and that required to produce symptoms. The ratio in 8a was 0.00025:0.0133::1:53; in 8b 0.0025:0.2::1:80. The discrepancy between these two ratios is due to experimental error.

In the same paper Coca and Kosakai were able to demonstrate that the reactivity of partially neutralized precipitin, as judged *in vitro* by the criterion of precipitation, is quantitatively the same as it is *in vivo* as judged by the criterion of anaphylactic shock.

The fundamental difference between the quantitative laws governing immunological and chemical precipitation is seen in a comparison of the experiments just described and the following: if to 10 cc. of a decinormal solution of silver nitrate 1 cc. of decinormal hydrochloric acid is added and if the resultant precipitate is removed a further precipitation can be produced by the second addition of 1 cc. of decinormal hydrochloric acid; moreover, the second precipitate will be of exactly the same weight as was the first.

The experiments of Coca and Kosakai reveal a simple but fundamentally characteristic peculiarity in the partial neutralization of precipitin which may be used as a test to determine the nature of human hypersensitiveness. If the latter is dependent upon the presence of precipitin, then the successive injection of identical or nearly identical quantities of the exciting agent should not cause repeated exhibition of symptoms. A multiple of the partially desensitizing dose is always required to cause symptoms upon a subsequent injection. Conversely, if symptoms, even in slight degree, recur upon such repeated injections, then precipitin can have no part in the production of the symptoms.

Applying this test to the observations of Alexander and of Mackenzie which we have referred to above, we must conclude that the serum sensitiveness which they were attempting to

modify was not anaphylactic in nature and that the reduced sensitiveness which they established was therefore not a partial desensitization.

In the first case cited from Alexander, two successive intravenous injections of 1 cc. of serum were followed in each instance by symptoms which were altered at the second injection but still marked.

In the second case six successive injections of nearly equal and large quantities of serum each produced symptoms.

In one of Mackenzie's cases an intravenous injection of 1 cc. of serum was given without symptoms, yet one and a half hours later 2 cc. caused marked reaction. The conditions in Mackenzie's second case were similar to those of the first and the results were the same.

One of the outstanding and practically important features of allergic hypersensitiveness is the specific reactivity of the skin. The easy accessibility of this tissue invited study of the effect of injections of the exciting agent of the hypersensitiveness upon the cutaneous reaction.

The writer had had numerous opportunities to make such a study following subcutaneous injections given in hay fever and asthma for therapeutic purpose. The effect of those injections has usually been a specific lessening of the general cutaneous reactivity, which, however, never approached extinction.

Recently Mackenzie and Baldwin (7, 8) have investigated the effect of local application of the exciting agent upon the cutaneous reaction and they have concluded that after repeated applications to the same site the reactivity of that site is specifically exhausted. In some instances the exhaustion did not persist longer than twenty-four hours; in one case, however, the intracutaneous injection of a 1:10 dilution of egg white caused a complete suppression of reactivity in the same site for three days.

In several unpublished experiments we had been unable to demonstrate an exhaustion of the cutaneous reactivity after an interval of about twenty hours following one or more intradermal injections, although in some instances the solution used for the attempted exhaustion was several times stronger than that used for the final test on the following day.

In further experiments, which we are about to describe, we have made repeated intradermal injections in the same site at short intervals and we have succeeded in producing practically complete exhaustion of the reactivity of the site to the substance in the particular concentration in which it had been injected. However this effect could be shown not to be specific; it appears, thus, to be merely a temporary exhaustion or fatigue of the general power of reactivity on the part of the tissues to irritation.

The experiments were carried out by the writer on himself. This circumstance permitted a continuous observation as to both the objective and the subjective phenomena during the considerable period of time occupied by the tests.

The writer is highly sensitive to horse and rabbit dandruff and also to the serum of these two animals. The first experiment was carried out with extracts of the danders; in the second experiment the two sera were used.

In both experiments the volume of fluid injected was always the same—0.01 to 0.02 cc. The concentration of the allergens in the different solutions is indicated by the quantity of nitrogen in 1 cc. of fluid as determined with the Kjeldahl method. For example, horse epithelium 0.01 means an extract of horse dander 1 cc. of which contained 0.01 milligram of nitrogen.

All injections were made intradermally and when repeated injections were made in the same site the needle was always introduced into the same puncture orifice.

EXPERIMENT 1[2]

July 23.

12:35 p.m.   Horse epithelium 0.01 is injected into site 1 on the anterior surface of the left forearm. 12:50 p.m. The area of reaction is drawn (fig. 1, chart 1.

4:55 p.m.   The wheal at site 1 has disappeared but the site is still red and there is an area of edema of the size of a quarter. Horse epithelium 0.01 is injected into site 1.

[2] On July 25 at the time the tests were made with horse epithelial extract 0.05 milligram of nitrogen per cubic centimeter there were noted some constitutional effects—coryza, asthma and slight urticaria. About five days after these tests were made there developed further mild symptoms of asthma with coughing and

5:05 p.m.   The area of reaction is drawn (fig. 2, chart 1).   The wheal is somewhat larger than that produced by the first injection and there is marked itching as at first.

10:15 p.m.   Site 1 still presents an area of redness and edema measuring about 2 by 3 inches.   Horse epithelium 0.01 is injected into site 1.

10:30 p.m.   The area of reaction is drawn (fig. 3, chart 1).   There is no increase in the zone of hyperemia.   A wheal is again formed accompanied by considerable itching.

July 24

8:15 a.m.   The zone of redness and edema still persists (2 by 3 inches). Horse epithelium 0.01 is injected into site 1.

8:30 a.m.   The area of reaction is drawn (fig. 4, chart 1).   The reaction as a whole is distinctly less than at the first injection.[3]

2:10 p.m.   There is very slight edema and no redness at site 1. Horse epithelium 0.01 is injected into site 1.

2:20 p.m.   The area of reaction is drawn (fig. 5, chart 1).   The wheal is of about the size of the earlier ones but it is of shorter duration; the zone of hyperemia is strikingly decreased.

4:10 p.m.   There is practically no edema and no redness at site 1. Horse epithelium 0.01 is injected into site 1.

4:25 p.m.   The area of reaction is drawn (fig. 6, chart 1).   The wheal is less sharply defined and hyperemia is almost absent.

6:20 p.m.   Horse epithelium 0.01 is injected at site 1.

6:30 p.m.   The area of reaction is drawn (fig. 7, chart 1).   The wheal is ill defined, merging into a small zone of hyperemia.

10:30 p.m.   Horse epithelium 0.01 is injected into site 1.

10:45 p.m.   The area of reaction is drawn (fig. 8, chart 1).   The reaction is distinctly more marked than it was at the last preceding injection.   There is no itching.

expectoration and an occasional hive. This necessitated the use of adrenalin in a dose of ten minims two to three times daily. The condition lasted for ten days and then subsided over a period of four days. After the second series of tests with serum to which the writer is less sensitive there was no constitutional reaction aside from an occasional hive during four to five days after the last test.

[3] It must be borne in mind that the relative duration of the reactions and the relative intensity of the hyperemia are not indicated in the drawings.

July 25

7:30 a.m.   There is a small papule ¼ inch in diameter about the puncture orifice. The papule is slightly tender and not red. Horse epithelium 0.01 is injected into site 1.

7:45 a.m.   The area of reaction is drawn (fig. 9, chart 1). The reaction is of short duration. There is no itching.

9:15 a.m.   The papule is slightly tender; there is no redness. Horse epithelium 0.05 is injected into site 1.

9:30 a.m.   The area of reaction is drawn (fig. 10, chart 1). The wheal is larger than the preceding one; there is no hyperemia and no itching. The wheal is of short duration.

10:40 a.m.   The papule is tender; there is no redness. Horse epithelium 0.1 is injected into site 1.

10:50 a.m.   The area of reaction is drawn (fig. 11, chart 1). The injection of this more concentrated solution caused an evanescent hyperemia and considerable itching which was felt also in the cubita fossa.

5:55 p.m.   Only the papule persists. Horse epithelium 0.1 is injected into site 1 and also into another place on the same forearm—site 2.

6:05 p.m.   The two areas of reaction are drawn (fig. 12a, chart 1—site 1; fig. 12b, chart 1—site 2). The reaction at site 1 was of very short duration without itching. That at site 2 was accompanied with itching, hyperemia and edema which persisted until 6:00 p. m. on the following day.

6:15 p.m.   Rabbit epithelium 0.05 is injected into the lower anterior surface of the right forearm—site 3.

6:30 p.m.   The area of reaction at site 3 is drawn (fig. 13a, chart 1). There is a lymphangitis which is visible at intervals from the injection site to the cubital fossa, where it is especially marked.

8:30 p.m.   The area of reaction at site 3 is again drawn (fig. 13b, chart 1). Both the wheal and the zone of hyperemia have increased. On the following morning edema and redness were still present.

8:35 p.m.   Rabbit epithelium 0.5 is injected into site 1.

8:50 p.m.   The area of reaction of site 1 is drawn (fig. 14, chart 1). There is a slight lymphangitis extending to the cubital

fossa and a very slight lymphangitis from the fossa to
the axilla.

10:45 p.m.   The reaction produced at site 1 by the last injection has
subsided; no trace of it is left.

July 26

4:00 p.m.   Horse epithelium 0.1 is injected into site 1.

4:15 p.m.   The area of reaction at site 1 is drawn (fig. 15, chart 1).
The resulting wheal was larger than that of 12a. It
persisted for one-half hour and was accompanied with
itching, which extended up through the cubital fossa
into the axilla.

EXPERIMENT 2

August 25

8:45 p.m.   Horse serum 0.1 is injected into site a on the right forearm

9:00 p.m.   The area of reaction is drawn (fig. 1a, chart 2).

8:50 p.m.   At site x on the same forearm rabbit serum 1 was injected
as a preliminary test of the cutaneous reactivity to that
material.

9:05 p. m.   The area of reaction at site x is drawn (fig. 1b, chart 2).

August 26

7:45 a.m.   Site a is still red and slightly edematous.   Horse serum 0.1
is injected into site a.

8:00 a.m.   The area of reaction is drawn (fig. 2, chart 2).   The reac-
tion appears to be slightly greater than after the pre-
ceding injection.   This is doubtless due to the per-
sistence of the effects of the first injection.

12:45 p.m.   Some of the wheal and of the hyperemia still persist
from the second injection.   Horse serum 0.1 is in-
jected into site a.

1:00 p.m.   The area of reaction is drawn (fig. 3, chart 2).

8:25 p.m.   There is still some hyperemia at site a.   Horse serum 0.1
is injected into site a.

8:35 p.m.   The area of reaction at site a is drawn (fig. 4, chart 2).
There is some itching in the site, but no increase in the
hyperemia and little in the wheal.

11:30 p.m.   Slight hyperemia still persists at site a.   Horse serum 0.1
is injected into site a.

11:40 p.m.   The area of reaction is drawn (fig. 5, chart 2). There is slight itching in the site but no real increase in the wheal nor in the hyperemia.

August 27

7:45 a.m.   Horse serum 0.1 is injected into site a.

8:00 a.m.   The area of reaction at site a is drawn (fig. 6, chart 2). There is very slight itching and faint hyperemia in the site. The wheal is not increased in size. There is an urticarial spot on the right thigh.

9:00 a.m.   Horse serum 1 is injected into site a.

9:10 a.m.   The area of reaction at site a is drawn (fig. 7, chart 2). This reaction lasted only twenty minutes. There was a constitutional reaction—asthma, with generalized itching and coryza with itching of the eyes—which persisted for about two hours.

12:10 p.m.   Horse serum 1 is injected into site a and into site b on the left forearm.

12:20 p.m.   The area of reaction at site a is drawn (fig. 8a, chart 2). This reaction lasted fifteen minutes. There was a constitutional reaction with coryza and generalized itching which lasted about two hours. Five urticarial spots appeared on the body.

12:25 p.m.   The reaction at site b is drawn (fig. 8b, chart 2). This reaction persisted for twenty-four hours.

3:00 p.m.   Rabbit serum 1 is injected into site a and into site c on the left forearm.

3:10 p.m.   The area of reaction at site a is drawn (fig. 9a, chart 2). There is no itching and no definite hyperemia. The size of the wheal is not increased. The reaction at site is marked, though not so intense as that at site b.

3:15 p.m.   The area of reaction at site c is drawn (fig. 9b, chart 2). The reaction persisted for about twenty hours, with edema, itching and hyperemia.

Both of these experiments bear out the conclusions which we arrived at in our discussion of the observations of Alexander and of Mackenzie: namely, that there is no true desensitization in allergy. In experiment 1, three successive injections of the same quantity of horse epithelium 0.01 produced marked reactions

(figs. 1, 2, and 3, chart 1). In experiment 2, two successive injections of horse serum 0.1 produced marked reactions.

The second noteworthy feature of both experiments is that after a site has been made nearly insensitive to one concentration of the material that is being injected, a vigorous reaction can be produced with a stronger concentration of the same material (fig. 11, chart 1; and fig. 7, chart 2).

In this fact may be seen a possible explanation of the belief of Mackenzie and Baldwin that the local "exhaustion" of the allergic reaction is specific. These authors may have tested the exhausted site with a second protein in a concentration higher than that of the protein used for the exhaustion.

The third result of the tests, which is evident in both experiments, is that the local insensitiveness produced by the repeated injections is not specific, as Mackenzie and Baldwin thought. It is merely the well known nonspecific fatigue of the tissues to protracted irritation.

Before this condition of fatigue has set in, the allergic mechanism continues to function upon repeated identical injections and even after the tissues have become fatigued to one concentration of the allergen the mechanism can be shown to be still intact by the injection of the allergen in greater concentration.

In experiment 1, after site (1) had been rendered insensitive to horse epithelium 0.1, the injection of the stronger extract of rabbit epithelium 0.5 produced a distinct reaction. This reaction was, however, much weaker in intensity and duration than that caused by the same injection into the fresh site (3). It was probably no greater than one that would have been produced in the "exhaused" site by horse epithelium 0.5.

In experiment 2, the drawings of the final reactions are in themselves convincing evidence of the non-specificity of the local insensitiveness produced by repeated injections.

The necessity of seeking further evidence to support the foregoing interpretation of our experiments has been recognized. It was necessary, first, to find out whether a non-specific local exhaustion of the skin is possible and, secondly, if this was true, to see whether any significant difference is demonstrable in the

development of this non-specific local exhaustion and of that induced by the repeated injection of allergens in specifically sensitive individuals. These two questions were investigated in some experiments that were carried out in this department by Dr. W. C. Spain and Dr. Ruth Guy with peptone and histamin.

The susceptibility of the skin to peptone was first described by Philippson in the Giorn. ital. d. mal. ven. e. d. pelle in 1899; that to histamin was first described by Eppinger in the Wien. med. Woch. in 1913. Since the local effect of both of these substances is non-specific they lent themselves well to the purpose in mind.

Mackenzie and Baldwin (8) state that Sollman found the histamin skin reaction inexhaustible and that they themselves found this reaction actually to increase in size and intensity with each successive application of the substance. A review of the experiments of Sollman (9, 10) shows that they are inadequate to determine this question. In the earlier experiments with Pilcher (9) he states (page 313), "When repeated applications are made (histamin) whether to the same or to the opposite arm, the wheals of the later applications appear smaller than the earlier." In his later study (10) Sollman describes only one experiment (no. 18) in which a "repeated" application of histamin was made at one site. In this experiment the second application of a $\frac{1}{10}$ per cent solution after a thirty-five minute interval produced a more vigorous reaction than had the first. No further applications were recorded. Evidently no information regarding the possible exhaustibility of the histamin reaction is obtainable from this experiment.

Mackenzie and Baldwin (8), also, used the strong $\frac{1}{10}$ per cent solution of histamin. They do not state how many applications were made nor what intervals of time were observed. In the experiments by Dr. Spain and Dr. Guy the concentration of the two substances in the solutions is given in percentage of the dry material. The quantity injected was the same as in the writer's experiment (0.01 cc.). All of the injections were intradermal. In the first experiment by Dr. Spain all of the injections were made into the same puncture orifice on the left forearm as follows:

July 23

12:55 p.m.	Histamin, 0.01.[4]
1:15 p.m.	Area of reaction drawn (figure 1, chart 3).
2:30 p.m.	Histamin, 0.02.
2:45 p.m.	Area of reaction drawn (figure 2, chart 3).
3:00 p.m.	Histamin, 0.04.
3:20 p.m.	Area of reaction drawn (figure 3, chart 3). The outlines of the wheal are indistinct.
4:00 p.m.	Histamin, 0.04.
4:20 p.m.	Area of reaction drawn (fig. 4, chart 3). The wheal forms slowly and is indistinct.
4:50 p.m.	Histamin, 0.04.
5:10 p.m.	Area of reaction drawn (fig. 5, chart 3). The wheal is not elevated above the level of the surrounding tissue; its outline is indistinct.
7:15 p.m.	Histamin, 0.04.
7:35 p.m.	Area of reaction drawn (fig. 6, chart 3). The outline of the wheal is questionable.
8:10 p.m.	Histamin, 0.04.
8:30 p.m.	Area of reaction drawn (fig. 7, chart 3). The outlines of the reaction are very indistinct: the surrounding skin is indurated in a space about 3 inches in diameter. There is no definite wheal.
8:30 p.m.	Histamin, 0.2. No definite wheal resulted from this injection. No drawing was made.

July 24

8:30 a.m.	The area of induration still persists, with a shot-like central node. No injection made at this time.
11:45 a.m.	Histamin, 0.4.
12:00 a.m.	Area of reaction drawn (fig. 8, chart 3). A very indistinct wheal is formed which is only slightly elevated. There is little erythema.

July 26

4:00 p.m.	Histamin, 0.4.
4:30 p.m.	The outlines of the reaction are very indistinct Area of reaction drawn (fig. 9, chart 3).

[4] 100 cc. of this solution contained 0.01 grams of histamin. The substance was obtained from Burroughs Wellcome & Co. under the name Ergamine Acid Phosphate.

In the second experiment by Dr. Spain, all of the injections excepting the final control injection were made into the same puncture orifice on the left forearm as follows:

September 13

9:00 a.m.   Histamin 0.1.
9:15 a.m.   Area of reaction drawn (fig. 1, chart 4).   There is decided erythema and much itching after five minutes.
10:00 a.m.   Histamin, 0.1.   The reaction caused by the first injection has subsided.   There is immediate itching.
10:05 a.m.   There is a sensation of dizziness and "light headedness." which lasts about one to two minutes.   Blood pressure is 112/80.   (The normal pressure is 118/80 –116/80.)
10:10 a.m.   Area of reaction drawn (fig. 2, chart 4)
11:00 a.m.   Histamin, 0.1.   The wheal and most of the erythema have disappeared.
11:15 a.m.   Area of reaction drawn (fig. 3, chart 4).   The reaction is apparently equal to the previous one.
12:05 p.m.   Histamin 0.1.
12:15 p.m.   Area of reaction drawn (fig. 4, chart 4).   There is very little reaction; the size of the wheal and extent of the erythema are not increased.
2:00 p.m.   Histamin, 0.1.
2:15 p.m.   Area of reaction drawn (fig. 5, chart 4).   There is little erythema and little increase in the size of the wheal.
3:45 p.m.   Histamin 0.2.
3:55 p.m.   Area of reaction drawn (fig. 6, chart 4).   There is some increase in the size of the wheal, though the reaction is not violent.   The zone of erythema is comparatively small.
5:00 p.m.   Histamin, 0.2.
5:10 p.m.   Area of reaction drawn (fig. 7, chart 4).   The reaction was slight.

September 14

9:00 a.m.   Histamin 0.2.
9:15 a.m.   Area of reaction drawn (fig. 8, chart 4).   The reaction was slight; the hyperemia was hardly visible.
9:30 a.m.   Histamin, 0.2.
9:45 a.m.   Area of reaction drawn (fig. 9, chart 4).   There is no increase in the size of the wheal; the hyperemia is vague.

11:00 a.m.   Histamin, 0.4.
11:15 a.m.   Area of reaction drawn (fig. 10, chart 4). The wheal
                 remains the same and the hyperemia is very slight.
11:00 a.m.   Histamin 0.4 in right forearm.
11:15 a.m.   Area of reaction drawn (fig. 11, chart 4).

The experiment by Dr. Guy was carried out on herself as follows:

September 22

10:29 a.m.   Histamin, 0.02, site 1.
10:37 a.m.   Area of reaction drawn (fig. 1, chart 5)
11:55 a.m.   Histamin, 0.02, site 1.
12:07 p.m.   Area of reaction drawn (fig. 2, chart 5).
 2:07 p.m.   Histamin, 0.02 site 1.
 2:40 p.m.   Area of reaction drawn (fig. 3, chart 5.) The wheal was
                 rather indefinite.
 4:00 p.m.   Histamin, 0.02, site 1.
 4:12 p.m.   Area of reaction drawn (fig. 4, chart 5). Wheal again not
                 sharply defined.

September 24

11:11 a.m.   Histamin, 0.02, site 1.
11:16 a.m.   Area of reaction drawn (fig. 5, chart 5). A definite
                 reaction.
12:50 p.m.   Histamin, 0.02, site 1.
 1:04 p.m.   Area of reaction drawn (fig. 6, chart 5). The reaction was
                 slight.
 2:09 p.m.   Histamin, 0.02, site 1.
 2:25 p.m.   Area of reaction drawn (fig. 7, chart 5). Slight reaction.
 3:56 p.m.   Histamin, 0.02, site 1.
 4:01 p.m.   Area of reaction drawn (fig. 8, chart 5). Slight reaction.
 9:08 p.m.   Histamin 0.02, site 1 right forearm.
 9:15 p.m.   Area of reaction drawn (fig. 9, chart 5). Slight reaction.
11:03 p.m.   Histamin 0.02, site 1.
11:17 p.m.   Area of reaction drawn (fig. 10, chart 5). Slight reaction.

September 24

10:47 a.m.   Histamin 0.02, site 1.
10:56 a.m.   Area of reaction drawn (fig. 11, chart 5). Very slight
                 reaction.
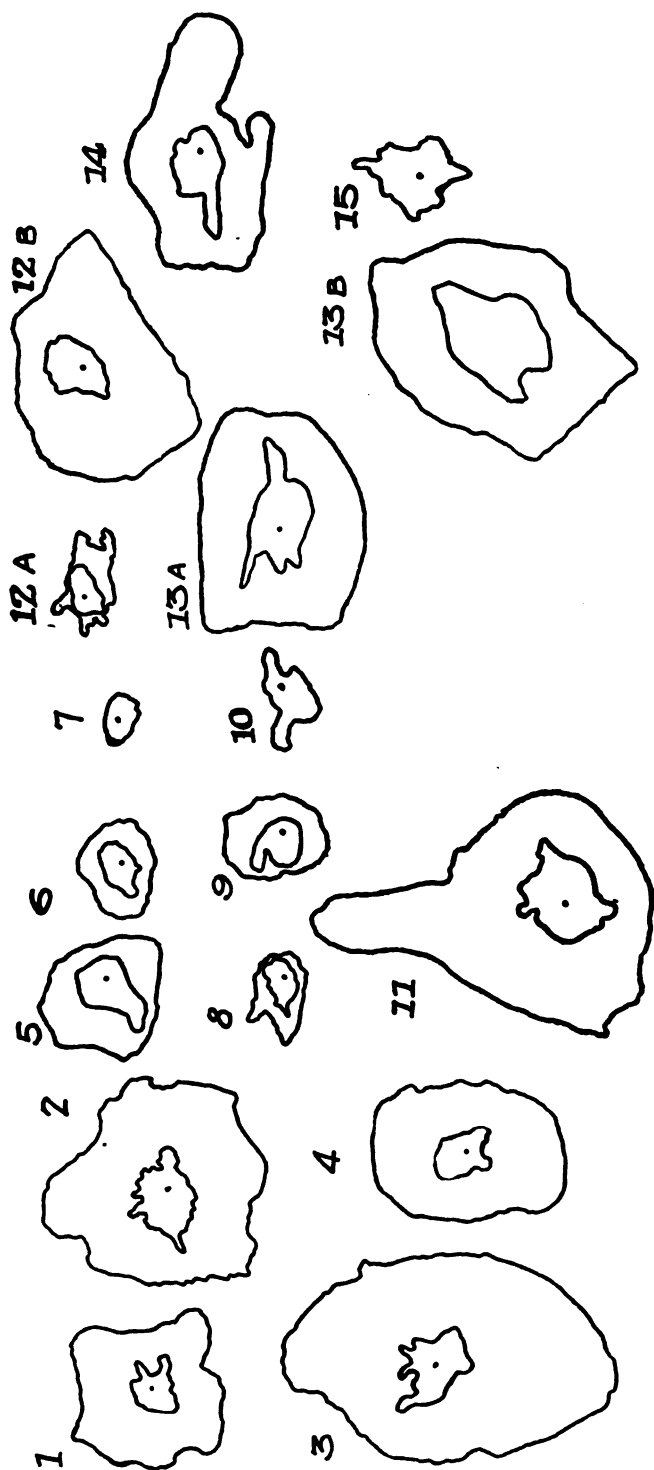
CHART 1

Fig. 1. Site 1; first injection; horse epithelium, 0.01.
Fig. 2. Site 1; second injection; horse epithelium, 0.01.
Fig. 3. Site 1; third injection; horse epithelium, 0.01.
Fig. 4. Site 1; fourth injection; horse epithelium, 0.01.
Fig. 5. Site 1; fifth injection; horse epithelium, 0.01.
Fig. 6. Site 1; sixth injection; horse epithelium, 0.01.
Fig. 7. Site 1; seventh injection; horse epithelium, 0.01.
Fig. 8. Site 1; eighth injection; horse epithelium, 0.01.
Fig. 9 Site 1; ninth injection; horse epithelium, 0.01.

Fig. 10. Site 1; tenth injection; horse epithelium, 0.05.
Fig. 11. Site 1; eleventh injection; horse epithelium, 0.1.
Fig. 12a. Site 1; twelfth injection; horse epithelium, 0.1.
Fig. 12b. Site 2; first injection; horse epithelium, 0.1.
Fig. 13a. Site 3; first injection; rabbit epithelium, 0.5.
Fig. 13b. Site 3; same reaction as in 13a, two hours later.
Fig. 14. Site 1; thirteenth injection; rabbit epithelium, 0.5
Fig. 15. Site 1; fourteenth injection; horse epithelium, 0.1.

The diameters of these drawings are exactly half those of the corresponding lesions.

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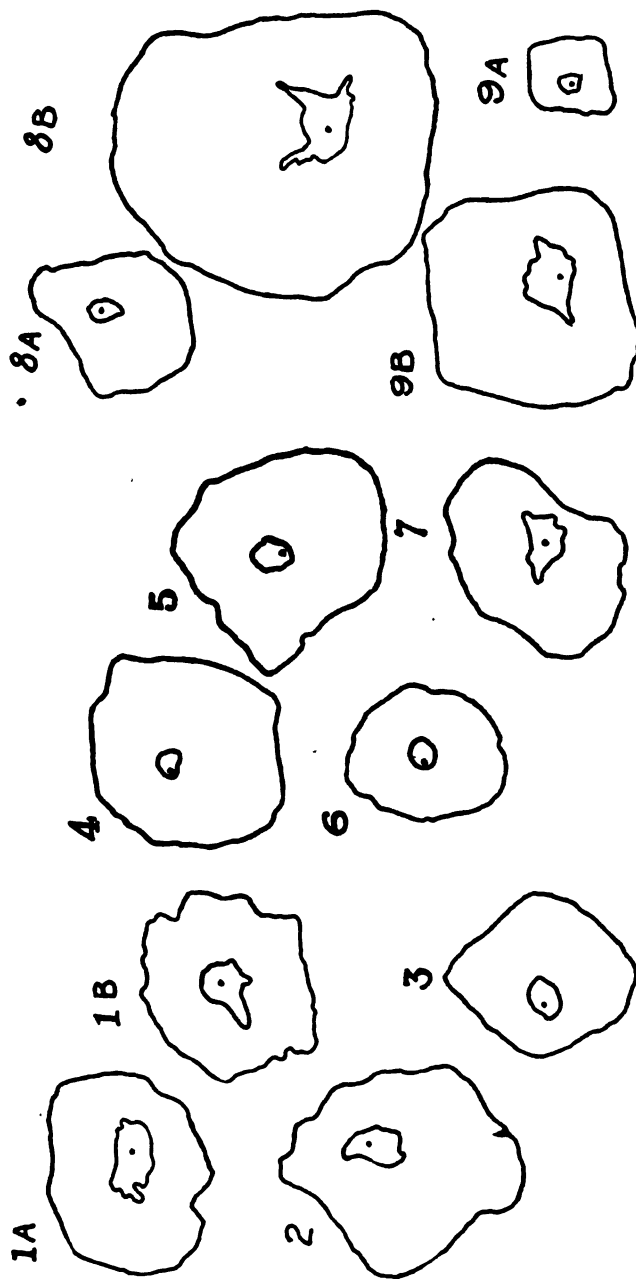
CHART 2

Fig. 1a. Site a; first injection; horse serum, 0.1.
Fig. 1b. Site x; first injection; rabbit serum, 1.
Fig. 2. Site a; second injection; horse serum, 0.1.
Fig. 3. Site a; third injection; horse serum, 0.1.
Fig. 4. Site a; fourth injection; horse serum, 0.1.
Fig. 5. Site a; fifth injection; horse serum, 0.1.
Fig. 6. Site a; sixth injection; horse serum, 0.1.
Fig. 7. Site a; seventh injection; horse serum, 1.
Fig. 8a. Site a; eighth injection; horse serum, 1.
Fig. 8b. Site b; first injection; horse serum, 1.
Fig. 9a. Site a; ninth injection; rabbit serum, 1.
Fig. 9b. Site c; first injection; rabbit serum, 1

The diameters of these drawings are exactly half those of the corresponding lesions.
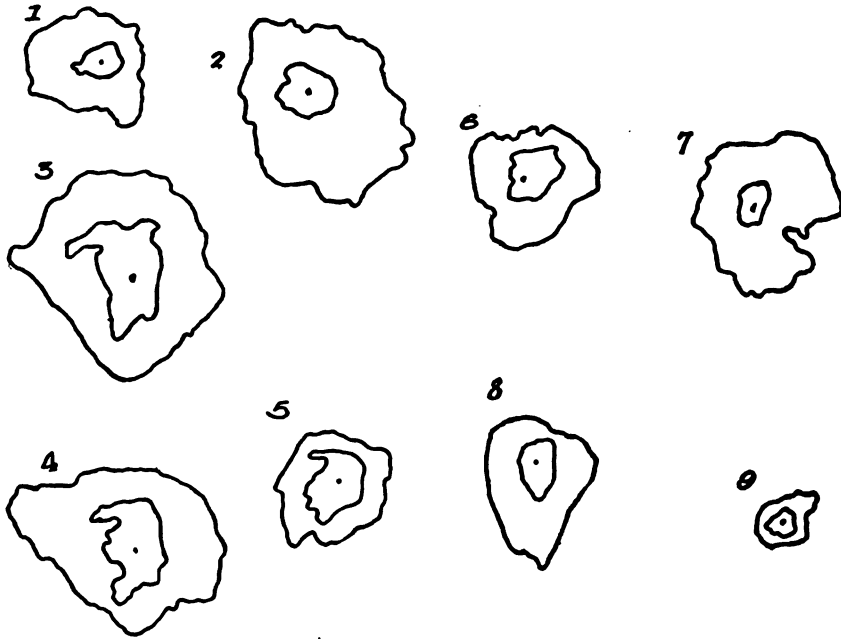
CHART 3

Fig. 1. Site a; first injection; histamin, 0.01 per cent.
Fig. 2. Site a; second injection; histamin, 0.02 per cent.
Fig. 3. Site a; third injection; histamin, 0.04 per cent.
Fig. 4. Site a; fourth injection; histamin, 0.04 per cent.
Fig. 5. Site a; fifth injection; histamin, 0.04 per cent.
Fig. 6. Site a; sixth injection; histamin, 0.04 per cent.
Fig. 7. Site a; seventh injection; histamin, 0.04 per cent.
Fig. 8. Site a; ninth injection; histamin, 0.4 per cent.
Fig. 9. Site a; tenth injection; histamin, 0.4 per cent.

The diameters of these drawings are exactly half those of the corresponding lesions.
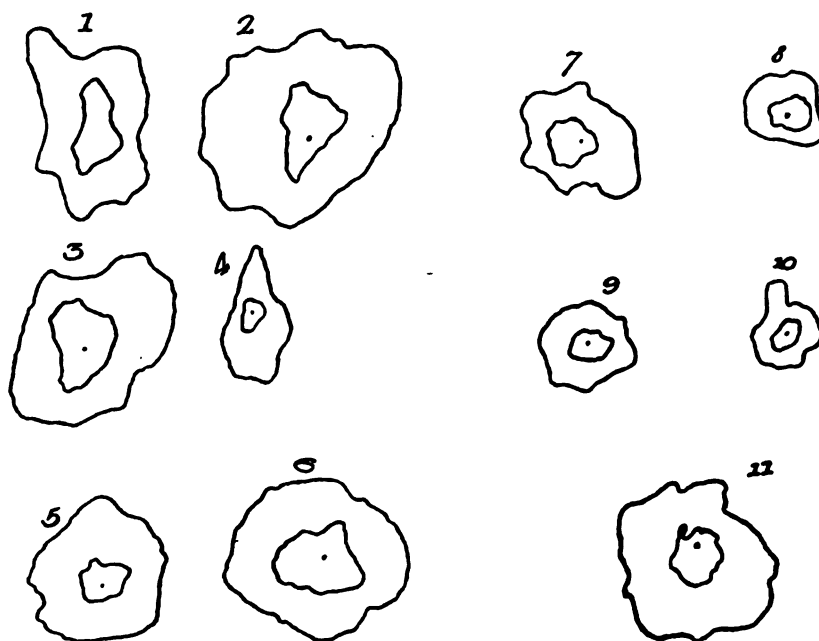
CHART 4

Fig. 1. Site a; first injection; histamin, 0.1 per cent.
Fig. 2. Site a; second injection; histamin, 0.1 per cent.
Fig. 3. Site a; third injection: histamin, 0.1 per cent.
Fig. 4. Site a; fourth injection; histamin, 0.1 per cent.
Fig. 5. Site a; fifth injection; histamin, 0.1 per cent.
Fig. 6. Site a; sixth injection; histamin, 0.2 per cent.
Fig. 7. Site a; seventh injection; histamin, 0.2 per cent.
Fig. 8. Site a; eighth injection; histamin, 0.2 per cent.
Fig. 9. Site a; ninth injection; histamin, 0.2 per cent.
Fig. 10. Site a; tenth injection; histamin, 0.4 per cent.
Fig. 11. Site b; first injection; histamin; 0.4 per cent.

The diameters of these drawings are exactly half those of the corresponding lesions.
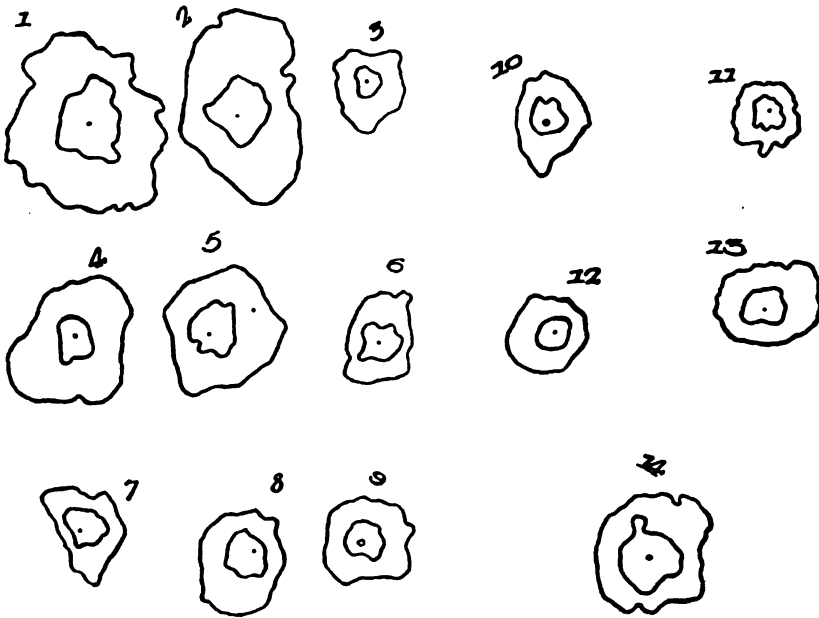
CHART 5

Fig. 1. Site 1; first injection; histamin, 0.02 per cent.
Fig. 2. Site 1; second injection; histamin, 0.02 per cent.
Fig. 3. Site 1; third injection; histamin, 0.02 per cent.
Fig. 1. Site 1; fourth injection; histamin, 0.02 per cent.
Fig. 5. Site 1; fifth injection; histamin, 0.02 per cent.
Fig. 6. Site 1; sixth injection; histamin, 0.02 per cent.
Fig. 7. Site 1; seventh injection; histamin, 0.02 per cent.
Fig. 8. Site 1; eighth injection; histamin, 0.02 per cent.
Fig. 9. Site 1; ninth injection; histamin, 0.02 per cent.
Fig. 10. Site 1; tenth injection; histamin, 0.02 per cent.
Fig. 11. Site 1; eleventh injection; histamin, 0.02 per cent.
Fig. 12. Site 1; twelfth injection; histamin, 0.02 per cent.
Fig. 13. Site 1; thirteenth injection; histamin, 0.2 per cent.
Fig. 14. Site 2; first injection; peptone, 5 per cent.

The diameters of these drawings are exactly half those of the corresponding lesions.

11:47 a.m.   Histamin 0.02, site 1.   Area of reaction drawn (fig. 12, chart 5).   Questionable reaction.

12:22 p.m.   Histamin 0.2   Site 1.

12:30 p.m.   Area of reaction drawn (fig. 13, chart 5).   Questionable reaction.

12:45 p.m.   Peptone (Witte) 5.0, site 1.

12:55 p.m.   No reaction.   No drawing was made.

12:45 p.m.   Peptone (Witte) 5.0, site 2, left forearm.

12:55 p.m.   Area of reaction at site 2 drawn (fig. 14, chart 5).

The materials used in the foregoing experiments by Dr. Spain and Dr. Guy produce their effect upon the skin by direct action and not by the mediation of an immunological reaction. This is evident from the fact that most individuals are susceptible to these substances upon the first administration of them. There are quantitative differences in this susceptibility which we may discuss in a future paper, but these differences do not contradict the principle just stated.

If the effect of the injection of the proteins of horse and rabbit dandruff is indirect—due to an immunological reaction—it should be expected that the existence of the intermediate immunological mechanism would be expressed in some difference in the results of the repeated injections as compared with those of the repeated injections of the directly acting histamin and peptone. However, a comparison of the five series shows them to be identical. In all, there is either an initial increase in the degree of the reaction or at least no change in it followed, then, by a decrease which is non-specific.

The evidence which we have presented seems to warrant the conclusion that the lessened sensitiveness induced in allergy by the injection of the exciting agent is a phenomenon which differs in its mechanism from that of desensitization in anaphylaxis. The former seems not to depend on the neutralization of precipitin as does the latter.

There is, however, to be considered the hypothetical possibility that the reactions of allergy depend on the presence in the tissues of "antibody-like" substances or so-called natural antibodies. This explanation was offered by Cooke, Flood and Coca (3)

but it was later dismissed by Coca (1), presumably on the ground that those hypothetical antibodies could not be neutralized completely.   We hold this ground to be for the present a valid one because there is no known natural antibody which cannot be completely neutralized.   It is useless to assume in allergy the existence of a natural antibody lacking a cardinal character of the known antibodies.   One may as well assume the intermediary action of natural antibodies to explain the specific physiological effects of drugs.

On account of the confusion that must result from the use of the well defined term "desensitization" to designate clinically lessened sensitiveness in allergy it is suggested that the latter be referred to as a "hyposensitization."   The etymological defect and also the convenience of this proposed term are the same as those of hypersensitization, which is in general use.

SUMMARY

1. The quantitative relations in partial desensitization are contrasted with those that obtain in the state of lessened sensitiveness in allergy, the differences being such as to indicate a difference in the mechanism of these two phenomena.

2. The phenomenon of "local exhaustion" of the allergic cutaneous reaction described by Mackenzie and Baldwin is studied and found, in disagreement with these authors, to be non-specific.

3. It is proposed to distinguish the lessened sensitiveness induced in allergy from the state of desensitization in anaphylaxis by designating the former condition as a state of hyposensitization.

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RHABDOMYOMA OF THE OVARY

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Tumors of striated muscle are sufficiently rare to make the observation of a new and characteristic case worthy of record. The rhabdomyoma which is the subject of the present discussion is of special interest because of the peculiar forms assumed by the myogenic cells and the wide variations of structure in the tumor.

At the present time a detailed discussion of the literature of this neoplasm is unnecessary; but the facts which are of more direct bearing on the present case may be mentioned. Benenati's list of 65 cases was published in 1903; and the cases reported since then do not show any material change in the relative frequency of this tumor in the various parts of the body. They may be divided according to the regions in which they occur. Rhabdomyoma is found most often in the genito-urinary tract. There are 39 cases occurring in this region: kidney, 13; testis, 9; uterus, 6; pelvis of the kidney, 3; vagina, 3; bladder, 3; ovary or uterus, 1; ovary, 2. The tumor of the ovary described by Virchow in 1850 was a papillary cystic rhabdomyosarcoma, some of the papillae being formed of striated muscle. A second rhabdomyoma of the ovary was reported by Vignard. It was similar to the tumor about to be described, the greater part being striated muscular tissue with cystic degeneration at one extremity.

Wolfensberger noted the frequency of this tumor in the neck and adjoining regions, which stand second to the genitourinary tract with 9 cases. These localities are: orbit, 2; temporal bone, nose, tongue, parotid, mandible, esophagus, and mediastinum. Four examples are found posterior to the pelvis. They were in the lumbar region, hip, ischial tuberosity, and anus.

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Homologous rhabdomyomas are found with greatest frequency in the heart, where 8 cases are recorded. Recently Wolbach has added an instructive case to the list. The remaining 5 examples were found in the following regions: pectoralis major, breast, shoulder, elbow, and thigh.

Muller has recently published a case in which rhabdomyosarcoma followed successive fractures of the femur. In his case the tumor probably arose from previously normal adult voluntary striated muscle cells. Such tumors, while probably not infrequent, are rarely reported, and belong to a group entirely different from the ordinary heterologous or teratomatous rhabdomyoma.

Clinically, rhabdomyomas are tumors of moderately rapid growth. Most of the tumors are well encapsulated and form no metastases; however, there are others with infiltration of surrounding tissues and recurrence after removal (Billroth, 2 cases; Buhl, Kaschewarowa) and some which formed metastases (Wolfensberger, Eberth, Benenati). Burgess has reported a case in which there were multiple metastases throughout the body.

Cohnheim's theory of embryonal rests has been accepted by many authors as accounting for the genesis of this tumor, especially of the heterologous rhabdomyomas. Ribbert has suggested as the origin of his renal tumor the development of striated muscle from the smooth muscle present in the pelvis of the kidney. His arguments do not seem to be convincing.

Benenati gives five possible derivations for rhabdomyoma testis. It may arise from smooth or striated muscle derived from the cremasteric muscle or from the gubernaculum; he finally concludes that the new growth arises from an embryonal rest. In 1904, Ribbert came to the conclusion that rhabdomyoma testis is a one-sided development of a teratoma. In the present communication I hope to extend this interpretation to rhabdomyoma of the ovary.

The genesis of teratoma has been variously explained. Verneuil in 1855 agreed with other writers in finding the origin of this tumor in a twin inclusion. Theories which found the source of tridermal neoplasms in polar bodies and isolated blas-
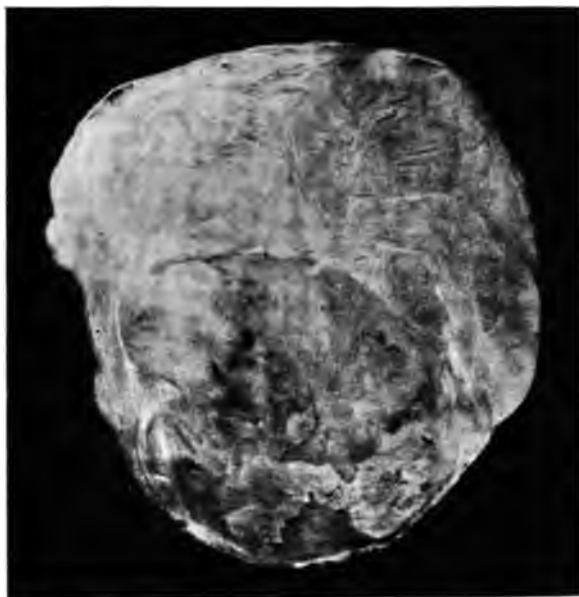
FIG. 1. GROSS SECTION OF TUMOR

Note capsule and thin strands of connective tissue surrounding the individual spheroidal masses which make up the tumor.

FIG. 2. LOW POWER FIELD, INCLUDING THE CELL SHOWN IN GREATER DETAIL IN FIGURE 5

The giant cells are surrounded by myxomatous tissue. In large portions of the neoplasm the latter element is found exclusive of any other.

tomeres have given way to one which is based on the partheno-
genetic development of the sex-cell. At the present time the
last mentioned theory has the greatest number of adherents.

The results of experiments performed by Stockard have led
me to believe that the twin inclusion theory will best explain
the origin of this neoplasm. In the following I shall endeavor to
prove that this theory is applicable not only to rhabdomyoma of
the ovary, but to most rhabdomyomas in common with many
simple tumors occurring inferiorly, either in front or behind the
pelvic zones, in the genitourinary tract, or superiorly, in the
neck region.

Present case. October, 1918, Edith M., one and a half years of age
was found to have a mass in the abdomen reaching half way to the
umbilicus. In shape and size it seemed to resemble a kidney with
the long diameter horizontal. It was freely movable. There were no
subjective symptoms. At the time of the operation nine months later,
the mass appeared to fill the whole abdomen up to the umbilicus. Early
in February, 1919, the child begun to vomit, to such an extent as to
cause the mother to give consent to operation. At that time a yellowish
discoloration appeared on the skin about the umbilicus.

Operation, performed February 9, 1919, disclosed a well encapsu-
lated tumor arising apparently from the region of the left ovary, fill-
ing the pelvis, with a narrower upper portion lying under the inferior
surface of the liver. The capsule was attached to the anterior ab-
dominal wall posterior to the umbilicus. The entire new growth was
removed. In the process of removing the tumor the capsule was
broken so that some of the myxomatous tissue fell into the abdominal
cavity. The child died of abdominal recurrence, May 31, 1919.

Gross anatomy. The tumor is covered by a thin, movable capsule
and consists of two parts, a lower, hard, spherical mass, 11 cm. in
diameter, and an upper myxomatous portion, 4 cm. in diameter. It
weighs 26 ounces. The surface is smooth and presents various
rounded protuberances. On section of the larger part, the capsule is
seen to run inward from the surface in thin strands throughout the
tumor, giving the impression that it was the covering of an ever in-
creasing growth. The tumor is made up of rounded masses, 3 cm. to a
few millimeters in diameter, suggesting that it grew by the appear-
ance of new parts, as well as by the increase in size of the older por-
tions. On section the myxomatous division was found to contain a
cavity.

FIG. 3. GIANT CELL WITH EXTENSION OF FIBRILLATED PROCESS IN MUSCLE
FIBER FORMATION

Adjoining are muscle fibers showing to some degree longitudinal and cross
striation.
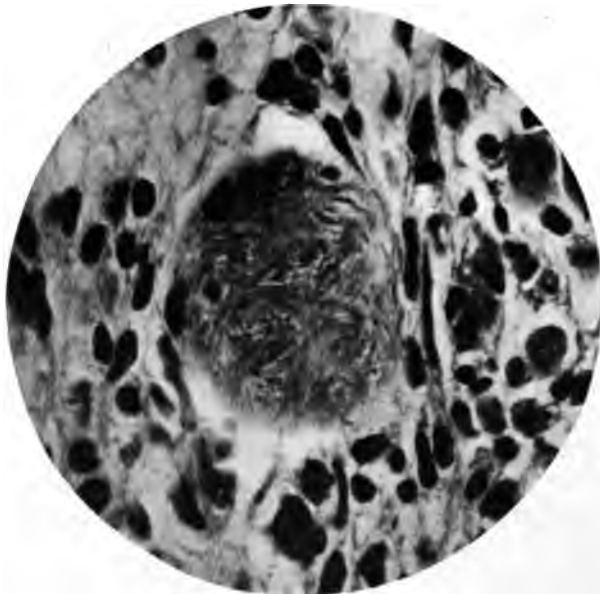
FIG. 4. GIANT CELL WITH WELL-FORMED FIBRILS ARRANGED WITHOUT DEFINITE
ORDER

There are four nuclei situated at one end of the cell

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Structure. Several main histological features deserve description. Perhaps the most striking are the *giant cells*. These cells have an acidophile cytoplasm in which usually there are concentric striae, most prominent at the periphery, while the perinuclear cytoplasm is granular. The nucleus or nuclei are round or broadly oval, sometimes containing 1 to 3 nucleoli. In other cells one or more centrosomes, as shown in figure 6, give evidence of active mitosis.

Muscle fibers. There are areas in the tumor composed of long cells with the characteristics of muscle fibers. The nuclei are oval and usually situated in the median axis. The peripheral cytoplasm is fibrillated, but only rarely can any cross striations be made out. Some of the fibers branch.

Myxomatous tissue. In several parts star-shaped cells with nuclei the size of those seen in the muscle fibers, and fibrils extending radially from them, can be seen separating the muscle fibers, showing that they developed alongside each other. In other places, they are the only element seen in large areas, giving the tumor a distinctly myxomatous appearance.

Histogenesis. The normal embryology of muscle may be taken as a guide to the anaplastic process. Striated fibers arise from myoblasts which elongate and by repeated mitotic division of their nuclei form a syncytium. The nuclei are surrounded by granular cytoplasm in which fibrils differentiate peripherally. The myofibrils became striated. The fibers increase in size and the nuclei migrate to the periphery. Heart muscle undergoes a similar development; but the nuclei remain centrally situated, sarcolemma never develops, and the individual syncytia are in connection with each other. Assuming that the tumor arose in a cell or group of similar cells we might expect to be able to follow the development till we arrive at the picture given in the histological description. Examining the three elements of the tumor it is possible to discern such an unfolding.

Giant cells. The giant cells as a group show a fundamental error in development; they have failed to elongate.

1. There are giant cells which show the extension of a process in an abortive development of muscle fiber (fig. 3).

2. There are giant cells which never elongated, while in other respects, they went through a more or less normal and complete development (fig. 5).

3. There are others which show increasing anaplasia until we come to some in which the fibrils lack definite order (fig. 4).
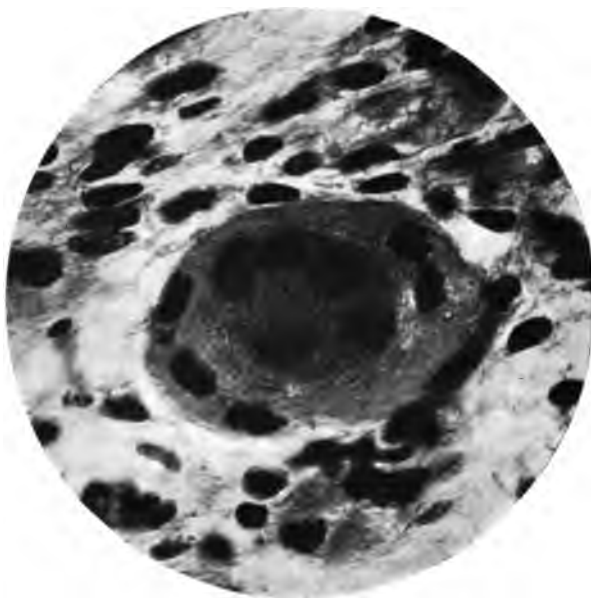
FIG. 5. THIS CELL DID NOT ELONGATE, BUT IN OTHER RESPECTS IT WENT
THROUGH A COMPLETE DIFFERENTIATION

The fibrils and cross striae are shown as concentric and radial striae
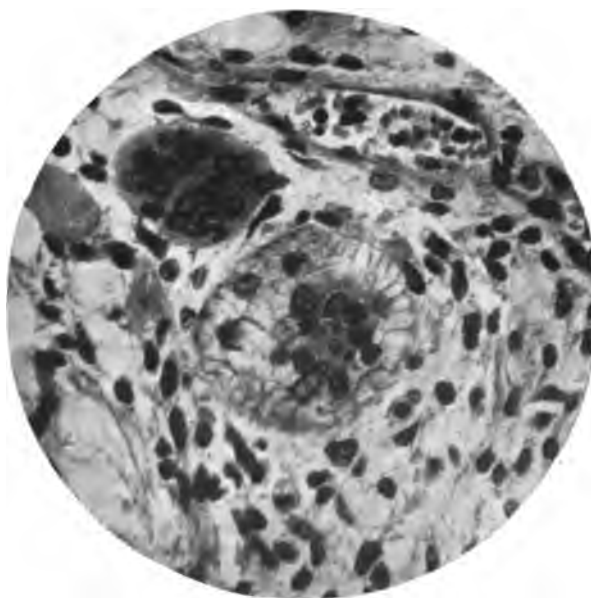
FIG. 6. ''SPIDER CELL'' IN WHICH THE CYTOPLASM BETWEEN THE PERIPHERY
AND THE NUCLEI HAS DEGENERATED AND BEEN ABSORBED

The perinuclear cytoplasm contains five centrosomes. This cell occurs only
in cardiac rhabdomyoma.

I am indebted to Mr. William Dunn for the photographs.

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4. Lastly, there is a group of cells, some of which resemble the "spider-cells," first described by Cesaris-Demel in a remarkable tumor, a rhabdomyoma of the heart (fig. 6). Others lie in spaces which Wolbach showed to be intracellular. Seiffert concluded that these spaces were formed within the peripheral differentiated portion of the cell by the degeneration and disappearance of the greater part of the remainder of the cytoplasm. The histogenesis of the "spider-cells" is similarly explained.

The fact that this type of cell has been found exclusively in rhabdomyoma of the heart, and the fact that I have found branching in some of the fibers, have led me to conclude that the present tumor arose from heart muscle. Katsurada, among others, reported heart muscle in dermoid cysts. It should be added that while sarcolemma is occasionally developed in rhabdomyomas, it is never found in those of the heart. Also, here as in heart muscle, the perinuclear cytoplasm remains undifferentiated. Some writers have described branching in other rhabdomyomas. This may indicate that in an anaplastic process such forms may be evolved, or that these other neoplasms had an origin similar to the one now being described.

The second element of the tumor need not long detain us. The muscle fibers are descendants of cells which developed more normally than those heretofore mentioned.

There are many cells which show intermediate stages of development between the muscle fibers and myxoma cells. The genesis of the myxoma is explained as follows: it occurred in the most rapidly growing portion of the tumor, and, as is well known, parallel with an increasing rapidity in the multiplication of cells there is a corresponding loss in differentiation.

In this laboratory there are three other rhabdomyomas with a mucous element very similar to the one described. The literature affords other cases in which myxoma complicated an otherwise pure rhabdomyoma (Billroth, Kaschewarowa, Targett). The possibility thus arises that with the preponderance of myxomatous tissue a rhabdomyoma may be misinterpreted as a pure myxoma.

In some parts of the tumor surrounded by myxomatous tissue, the nuclei disappear, leaving areas of pure fibrils. These areas of fibrils raise the question of the possibility of the multiplication of fibrils without the corresponding multiplication of the myxoma cells. An analogous condition seems to obtain in neurocytoma, in which a similar picture of excessive fibrils may appear. It does not seem possible for the multiplication of fibrils to take place without the corresponding division of nuclei, but the overgrowth of fibrils of the individual cells is quite possible. A similar overgrowth of cytoplasmic derivatives is seen in the formation of epithelial horn.

Origin of the tumor. 1. Having shown that all the elements may be derived from myogenic cells, the next question to be considered is the origin of the group of cells from which the tumor arose. In considering this heterologous neoplasm we may leave a metaplastic origin out of the question. This leaves two possibilities: the tumor arose (a) from an embryonal rest; or (b) as a one-sided teratoma.

We may safely eliminate the former possibility. In general, nothing is known about embryonal muscle rests in the ovary. In particular, the organ is not situated near striped muscle so as to make such a rest possible. This conclusion becomes more evident since we are dealing with cardiac muscle.

It is an accepted fact that one element of a teratoma may outgrow the others (Pick and Walthard) and that the neoplasm may be represented by a single tissue. In the case of rhabdomyoma this conclusion gains added strength when we remember that striated muscle sometimes identified as cardiac, is often found in teratomas. It is interesting to note that Cornil thought the rhabdomyoma described by Vignard, the only case in the literature similar to the present one, was a fetal inclusion.

Additional support is found in the occurrence of similar tumors in an analogous organ. Rhabdomyoma testis is admittedly of teratomatous origin.

2. Since the teratomatous origin is the only one which will explain the rhabdomyoma it is necessary to determine in what manner the teratoma arose, and again there are two possibilities; it arose either from a sex cell or from a twin inclusion.

In the ovary, where there are totipotent sex cells, it may seem unnecessary to seek further for the origin of the teratoma. The commonly accepted explanation is the parthenogenetic development of this cell. This view point is open to criticism because mammalian ova, with the exception of one series reported by L. Loeb, have never been observed to develop parthenogenetically and then continue a separate existence. Loeb, himself, claims little vitality for these ova, while Bandler and Wendeler quote other investigators to the effect that ova which undergo such a development invariably end in complete degeneration.

On the other hand, the twin inclusion theory has a basis in experiments recently performed by Stockard. By slowing the rate of development of fish embryos at the time when the primary bud should arise, two or more buds may develop simultaneously. The degrees of doubleness of the resulting individual is determined by the distance between the two buds on the blastodisc. At 180°, that is diametrically opposite each other, two complete individuals are formed.

When one bud gets the start of the other by any advantage it obtains a supremacy which allows it to develop into a perfect individual. The component arising from the inferior bud is suppressed and interfered with so that it develops abnormally. The twins are attached anteriorly in the head and neck region, or posteriorly, the great majority of them in the latter situation. There are all degrees of this inhibition of the second twin until it is reduced to a mere fragment of included tissue. The point made by Stockard is that human monsters have been born which show exactly the same relation to each other, as above described. Monsters attached superiorly on the head, neck, or upper thorax, or inferiorly near the sacrum, or anteriorly in the ventral region, can be traced in an unbroken series until the inhibited twin is represented by a teratoma or dermoid.

On the basis of these experiments it is possible to explain the presence and frequency of rhabdomyomas in the regions given in the first part of the paper, that is, 9 superiorly in the neck region, 43 inferiorly in the pelvic region.

No facts have been brought forth to disprove the twin inclusion theory. However, there are two questions which need further discussion. The first is the great frequency of teratomas in the sex glands; and the second, the occurrence of chorioma in males and in females at such a time as to shut out the possibility of impregnation. These phenomena can be explained by the twin inclusion theory.

Bonnet remarked the fact, in defending the theory of the origin of teratoma from an independent blastomere, that if a blastomere should separate from the remainder of the blastula, the organ to which it would be most likely to attach is the mesonephros.

For a long time the mesonephros is the largest organ in the abdominal cavity. It is the most vascular. Later the mesonephros survives as functional and vestigial parts of the genital system; in the male, the efferent ducts, the paradidymis, epididymis and vas; in the female the paroöphoron, parovarium, and Gartner's ducts.

If the twin were attached to the mesonephros, we should expect to find it adherent in the testis to the mesonephric tubules which join those of the sex gland. This is actually the case, for Ewing in his paper on teratoma testis points out that almost invariably the neoplasm arises in the neighborhood of the rete. We can explain those which occur in the scrotum external to the tunica albuginea by the accidental attachment to other portions of the mesonephros. Bandler has made an attempt to explain teratoma as a development of the paroöphoron. We now see that while it does arise from this region it is not a development from this organ, but is rather attached to it.

The position of teratoma is explained by the organogenesis of the sex glands in which the mesonephros plays such a prominent part. This accounts for most teratomas attached anterior to the pelvis. It explains the 39 rhabdomyomas found in the genitourinary system. The other four, being situated posteriorly, were not able to become adherent to the mesonephros. From these facts arises the thought that many simple tumors of the ovary may be of teratomatous origin. Ewing has come to

the conclusion that practically all common tumors of the testis are of similar origin.

In taking up the question of chorioma, we may again turn to an observed fact. Up to the present time nobody has been able to cause the spermatozoa of any species to develop into an embryo. How then can we explain chorioma testis except as an early inhibited twin with the predominance of this single chorionic tissue?

Boestrom reported a case of chorioma with multiple metastases throughout the body in which the testicles were normal. Djewitski recorded a case of chorioma of the bladder in a virgin, seventy-five years old. Surely rather late for the parthenogenetic development of an ovum, but not against the growth of a long repressed twin. The facts of extragenital chorioma can be explained best as a twin inclusion. The relations between the two components is such that with the growth of one there is an inhibition of the other. The rate of development of the host after birth is much retarded, and the repressed component is given the required opportunity to grow. This explains why the majority of teratomas begin their growth in early life rather than during the longer adult period. Senile atrophy of the host gives the parasite a last chance for development.

Causal genesis. The frequency of teratoma testis after trauma is well known. This may not only directly precipitate the rapid growth, but indirectly, by lowering the vitality of the organ, give the long repressed twin inclusion the temporary advantage. The real causal genesis lies in the potential energy for growth constantly waiting a chance for expression. Further, that the growth should be autonomous is the only possibility in tissues which have been so disorganized. This may be capable of experimental verification.

To sum up the points of the theory of the twin inclusion: It is based on observed facts and therefore can give a true casual genesis. It explains the position and frequency of teratomas, in the sex glands as well as elsewhere in the body, obviating the necessity for multiple explanations for teratomas. The theory holds for all one-sided teratomas.

The possibility of new evidence in favor of any other explanation for the frequency of teratomas in the sex glands will not disprove the twin inclusion theory, but will be complementary to it. Likewise the theory holds independently of the method of the formation of the twin.

According to the theory of twin inclusion, the complete sequence of events is as follows: an ovum is fertilized; through some unfavorable condition the rate of development is slowed; two primary embryonic buds are formed on the blastodisc, one having the advantage over the other. The favored bud develops into a perfect individual, during which process the smaller bud is inhibited and disorganized. The latter, being united to the more vigorous component at its ventral region, becomes adherent to the mesonephros; hence, later it forms a part of the ovary. With the birth of the child and its consequent diminished rate of development, the inhibited twin is given a new opportunity to grow. Through its previous disorganization it is not capable of orderly development. The heart muscle grows more rapidly and succeeds in crowding out the other elements. The growth gains in momentum, and myxoma cells are formed which have no resemblance to the earlier muscle cells.

SUMMARY

The case described is one of rhabdomyoma of the ovary in an infant.

Three elements arose from one tissue by (a) histogenesis more or less normal; (b) anaplastic development; (c) degenerative changes. Although only one tissue, cardiac muscle, is present, the tumor is of teratomatous origin. Many simple tumors of the ovary are probably of similar origin. Many tumors of the head, neck, thorax, and genitourinary and posterior pelvic regions are of teratomatous origin. This group includes the great majority of heterologous and some of the homologous neoplasms.

Teratoma is a twin inclusion. A group of cells is found in this tumor which appears only in rhabdomyoma of the heart.

Therefore, the present tumor is a rhabdomyoma of the heart of a twin inclusion. Fibrils may be produced in such overabundance as to lose connection with their nuclei and seem to be multiplying independently. Myxoma may be secondary to rhabdomyoma.

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TERATOMAS AND THEIR RELATION TO AGE

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The great accumulation of reported cases of teratomas offers an excellent opportunity to review the data, with the object of investigating the relation of their frequency to the age of the host. In the course of the study, it has become apparent that this relationship is so definite as to assume the form of a general law.

The largest collections of teratomas were gathered by Taruffi and Ahlfeld. Both of these observers stressed the large congenital forms which are situated in the head, the thoracic, the abdominal, or the sacral regions. Gonadal teratomas occur most frequently in early adult life (Wilms). Among others, Askanazy investigated the internal craniopagi, and Ekehorn, the internal thoracopagi. Lexer and Nakayama studied the abdominal inclusions and pygopagi.

Though many hypotheses have been advanced on the origin of teratomas, they may be resolved into two view points: The teratoma is either the offspring or the twin of its host. Stockard has recently produced experimental evidence in favor of the latter conception.

CRITERIA AND METHODS OF STUDY

Two precautions have been observed in compiling the present statistics: (1) Only growths of tridermal or bidermal origin have been considered. No such case has been omitted. (2) Special effort has been exerted to determine the age at which the teratoma began its growth. The first increase in size of external growths may be accurately observed. For those

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situated internally, the initial symptom was used as an indicator. Where the history was deficient, the age at which the operation took place or at which death occurred was taken as the closest approximation obtainable. The last criterium particularly applies to the teratomas of the aged. In this manner 895 cases have been studied. Sometimes a period of slow growth is followed by one of heightened activity. Such is the case of chorioma testis reported by Jackson, in which growth commenced at the age of twenty and slowly continued to twenty-three, after which the increase in size became extremely rapid. Since the relationship between the growth of the host and that of the teratoma is of interest, in such cases, the beginnings of both periods have been noted. A similar effort was made for internal teratomas, thus bringing the total number of growths tabulated to 975.

In systematizing the results it was noted that though the variation from year to year is considerable, there seems to be an orderly waxing and waning of the number of cases to an extent which justifies the drawing of a curve. An average has been drawn in order to minimize accidental variation. Six year periods have been chosen because they are the longest which correspond to actual changes throughout the length of the curve. The first period begins at fertilization and ends at five. In the curves which are drawn to a scale of one-half, the abscissae represent the age of the host when the tumor began its growth, the ordinates the number of cases in each year.

THE CURVE FOR ALL TERATOMAS

After an initial maximum rise, the curve falls and remains low from five to eleven years (fig. 1). At eleven it achieves a higher level, which is increased at seventeen and twenty-three years. The second maximum is found between twenty-three and twenty-nine years. The curve falls gradually at twenty-nine and thirty-five years, and then more rapidly at forty-one, after which it becomes progressively lower towards its end at seventy-six years. Eighty-seven per cent of the teratomas occur before forty-one years and 95 per cent before fifty-three.

Most congenital teratomas do not evince postnatal growth and since this study concerns only those which do grow, for the early maximal total may be substituted the smaller number of tumors, showing power for growth, represented by the lower broken line in the graph.   With this correction the highest point in the curve is found between twenty-three and twenty-nine years. This is borne out by Wilms who finds the period of greatest frequency for sex-gland teratomas to be between the ages of twenty and thirty years.   His conclusion is to be expected because teratomas occur most frequently in sex glands.
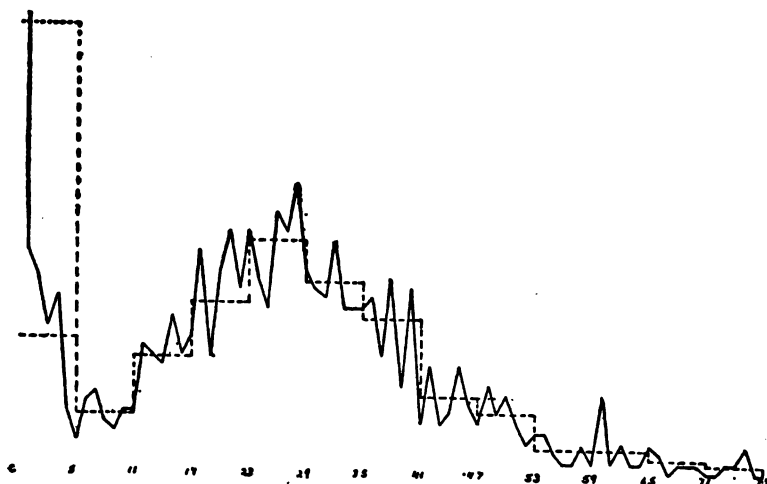
FIG. 1. CURVE FOR ALL TERATOMAS

The abscissae stand for the years of appearance of growths, the ordinates for the number in each year.   Both are drawn to a scale of one-half.   The total number of congenital tumors is not shown.   The dash line indicates the average for each six-year period.   In the first six years there are two; the upper one stands for all cases, the lower for those which had power of postnatal growth. The mode is from twenty-three to twenty-nine, the time when growth stops.

STUDY OF THE CORRECTED CURVE

There are three aspects of the described phenomenon: First, it is evident that the total number of teratomas at any age increases with the actual growth of the individual.   As size

increases, the total number of teratomas increases. But it must be remembered that while size is increasing, growth rate is falling. Therefore, second, teratomas become more frequent as growth slows down, at the time when growth potential becomes smaller. Growth must be recognized as involving two elements: increasing actual proportions, and decreasing growth potential. Hence, third, the total number of teratomas at any age increases as growth potential diminishes.

When one recalls the fact that the changes in growth rate are not constant, the number of teratomas is seen to bear even a closer relationship to the growth of the hosts than has been indicated. Not only do these tumors appear as growth of the host slows, but during their time of appearance the teratomas are more frequent in the periods of slower growth of the host. Teratomas are common in early infancy following a space of the most rapid proliferation of all—fetal growth. They increase again when the comparatively rapid growth rate of early childhood gives way to the slower one of pubescence and lastly they are found in greater numbers as active growth gradually ceases.

To explain the relative number of teratomas appearing in the several periods we must take into consideration an additional factor, the growth potential of the embryonal rest, for a teratoma by most theories arises in an embryonal rest of some kind. It is known that the great majority of embryonal rests do not grow; they either degenerate or remain dormant. Others achieve a more or less perfect adult growth, while a few develop into tumors. Tridermal rests act in a similar manner. Thus we have a few with high growth potential, many with less capacity for growth, and finally others which remain latent unless they are stirred to development by an external stimulus.

During the first six years of life the growth rate of the host declines rapidly, his growth potential is greatly reduced, and the number of teratomas of relatively higher potential, capable of proliferation, is comparatively large. From five to eleven years the growth rate is fairly constant, the loss of potential is small, and the additional number of teratomas released is few. The next appreciable change takes place during the differentia-

tion occurring at puberty and accompanying the maturation of the sex organs. Here the number of teratomas begins to increase and continues to do so until growth finally stops, at which time the greatest number of teratomas make their presence known. Although the loss of growth potential in the host becomes smaller, being least in the final period from twenty-three to twenty-nine years, yet just because growth itself is slow, there is an ever increasing number of embryonal rests of low potential, capable of expressing their latent growth energy. The very inactive rests even at this period do not have sufficient energy to start growth spontaneously and are therefore considered in another group at a later time. However, they may serve as a nidus for neoplasms since many do commence development after the growth of the host has stopped.

Thus the number of teratomas appearing at any given time depends upon the amount of loss in growth potential of the hosts during that time and the number of rests whose potential is large enough to proliferate under these conditions. Evidently *the number of teratomas in any period varies inversely to the growth potential of the hosts and directly as that of the embryonal rests.*

ANALYSIS OF CURVES OF AGE INCIDENCE OF TERATOMAS IN THE DIFFERENT LOCATIONS

The object of this study is to show the relation to the general law of the occurrence of teratomas in the various situations.

Thoracopagi. Ekehorn in his collection of teratomas of the anterior mediastinum finds fifteen cases occurring between the ages of twenty and thirty, four between thirty and forty years, and four more to sixty, thus agreeing with the present curve in showing the maximum occurrence from twenty-three years to twenty-nine.

Abdominal inclusions. The abdominal tridermal growths are frequently discovered at birth but continue to be found throughout life, with a second rise from twenty-three to twenty-nine years.

Craniopagi and pygopagi. There is not a sufficiently large number of head and sacral teratomas to yield reliable statistics.

Statistics of various authors.   They show a close agreement with original statistics here presented.   Note increased number of dermoids during pubescence

THORACOPAGI (CHRISTIAN)		DERMOIDS (PAULI)		EMBRYOMES (CHEVASSU)		SEMINOMES (CHEVASSU)		CARCINOMAS		
									Testis	Ovary
10–20	1	1–5	4	0–5	5	0–19	0	0–5	2	11
20–30	19	5–10	3	6–17	0	20–25	1	5–11	2	25
30–40	7	10–15	10	18–24	16	25–30	8	11–17	1	48
40–50	2	15–20	8	25–29	11	30–35	8	17–23	12	19
50 +	2	20–25	12	30–34	11	35–40	19	23–29	29	26
		25–30	14	35–39	7	40–45	14	29–35	30	21
		30–35	10	40–44	3	45–50	4	35–41	34	16
		35–40	11	45–49	4	50–60	2	41–47	27	16
		40–45	8	50–59	2	60–63	2	47–53	10	14
		45–50	10	60–75	2			53–59	8	14
		50–55	5					59–65	1	1
		55–60	3					65–71	1	
		60–65	1							
		65–70	2							
		70 +	2							
	31		103		61		59		157	207

Inclusions according to age and situation.   Those which are capable of growth appear in greater number from twenty-three years to twenty-nine years

AGE	CRANIOPAGI	THORAC-OPAGI	ABDOMINAL PARASITES	OVARIAN TERATOMAS	TESTICULAR TERATOMAS	PYGOPAGI	TOTAL
—5	78	55	36	17	48	45	279
5–11	1	3	6	15	7	1	33
11–17	4	11	18	27	9	1	70
17–23	4	14	16	44	32	4	114
23–29	1	16	20	52	55		144
29–35		10	15	47	44		116
35–41		4	8	39	43		94
41–47		4	4	22	15		45
47–53		4	4	19	7	2	36
53–59		1	1	13			10
59–65		1	2	10	3		16
65–71			1	6	1		8
71–77	2		1	2			5
Total. . . .	90	123	132	313	264	53	975

Most are congenital. The internal craniopagi occur most frequently from eleven to twenty-seven years. The only year where there is more than one case is the nineteenth, where there are two. The last teratoma capable of spontaneous growth appeared at twenty-seven. The later examples of sacral teratomas are even rarer than those occurring in the head. There is one each at ten and thirteen years and four from nineteen to twenty-three.

Ovarian teratomas. Turning next to the gonadal teratomas and comparing the testicular and ovarian curves, we see that the latter is less variable (fig. 3). This is due to the delayed diagnosis of so many of the ovarian teratomas, probably because of their slow growth, as their structure is often of the adult type. Their internal position further postpones their discovery, yet dermoids are sometimes found by accident. Nevertheless, the largest number of tumors occurs between the ages of twenty-three and twenty-nine. From a review of one hundred and three cases Pauli finds dermoids appearing most often from twenty to thirty years.

Another difference between the ovarian and testicular curves is the greater rise in the former in the two periods between eleven and twenty-three years. This phenomenon might be expected as a result of the growth differences since, in the male, postpubescent growth is more rapid than in the female.

Teratoma testis. The external situation of the male sex gland allows prompt discovery of its tumors. Although the greatest decline in rate of growth occurs early in life from birth to four years and the number of testicular teratomas at this time is large, the maximum number nevertheless occurs at a later period. This is probably due to the fact that growth under four years is comparatively very rapid in spite of its fast declining rate. The modal year of the curve is twenty-six. It is interesting to note that the last growth cartilage of the long bones ossifies at twenty-five.

Teratomas in the testes commence growth most frequently between the ages of twenty-three and twenty-nine years. Chevassu finds the maximum between twenty and thirty years (fig. 2).

TERATOMAS OF LATER LIFE

After establishing the time of greatest frequency of teratomas, it still remains to account for those of old age. Those diagnosed after the age of fifty-one may be divided into two groups. The first consists of neoplasms of adult structure which had reached the limits of their capacity for post-natal growth, while those of the second are more malignant. To the first group belong such tumors as the following:

Craniopagi. Beck reports a case in which a dermoid was found at autopsy in place of the hypophysis in a woman seventy-four years of age. Eberth reports a similar accidental finding beneath the dura in a woman of seventy-five.

Thoracopagi. There are two examples in Ekehorn's collection—Pinder's case of a patient with bulbar paralysis, aged fifty-three, in whom the dermoid was discovered at autopsy; and Lebert's of a man of sixty who had been dyspneic since his sixteenth year.

Abdominal inclusions. Rizzoli (Taruffi) reports two cases of late abdominal inclusions, one at sixty, the other at sixty-two. Symptoms had been present for a long time in both. In one of the cases they appeared first at the age of twenty.

The tumors mentioned thus far were benign, though some produced symptoms because of their size and position.

In the second group of neoplasms the element of trauma becomes important in the etiology. There is Bonney's report of a retroperitoneal chorioma of a man of sixty-seven, and Goebell's of an abdominal teratoma that became malignant at fifty-four, twenty-seven years after a mass had been diagnosed. Djewitski reports a chorioma of the bladder which first gave symptoms at the age of seventy-three. The same irritation which produces a papilloma of the bladder may transform an otherwise benign embryonal rest.

Pygopagi. Hudson describes a sacral teratoma which began growth at the age of fifty-two; the history shows that a nodule had existed in that region since the birth of the patient. The histological picture is one of a tridermal rest with cancerous degeneration of mucous glands. It is similar to an old age

cancer arising in previously normal tissue. Evidently in the last four cases it is not growth potential of the embryonal rest but an extrinsic traumatic influence, to which every part of the body is subject, that caused the proliferation of cells. A case of Briddon's beautifully illustrates both these factors occurring in the same growth but independently and at different times. It concerns a sacral dermoid which appeared externally at the age of twenty-two and then ceased growth till the fifty-second year, when it underwent epitheliomatous change.

Teratoma testis. There are three examples of late teratoma testis. Lexer quotes one from v. Bergmann's clinic in a man of sixty. On section the growth was of adult structure.

Ewing and Pepere report cases which first showed growth at the ages of sixty-one and sixty-three, respectively. The microscopic examination in both instances showed carcinomatous change of one element in a totipotent rest.

Ovarian teratomas. Of thirty-two dermoids, thirteen exhibited malignant transformation of a carcinomatous, sarcomatous, or endotheliomatous type. Four showed thyroid structure, of which three were rapidly growing tumors. In six reports details were lacking. However, since the growths were called dermoids, their structure must have been of the adult type, like that of the remaining nine inclusions.

To summarize, the late appearance of stationary teratomas is due to their delayed discovery, while that of growing teratomas is caused by their injury.

Carcinoma testis. Carcinoma testis is discussed in this place not only for its possible teratomatous origin, but because traumatic etiology links it with the tumors of later life. In many cases of teratoma testis in young people the transformation of a slowly into a more rapidly growing tumor is caused by trauma. In older people the growth is rapid from the start. The same sequence of events obtains for carcinoma testis. The cell of many cases of carcinoma testis is characteristic, with a large nucleus and clear cytoplasm. Sometimes the growth is called a sarcoma; the difference in opinion is due to the fact that no analogous cell is found in the human body. This same cell is

often found with teratoma testis. There are only two probable interpretations: (1) The irritation caused by some extrinsic factor, in this special case, by the teratoma on the tubule cells, is the cause of carcinoma. (2) The unique type of cell is of teratomatous origin. Chevassu takes the position that it develops from the adult spermatogonia, putting the tumor in the class of acquired carcinomas. The final convincing link in the chain of evidence has not been produced, for he has not been able to trace the steps of anaplastic change from the spermatogonia to the carcinoma cell.

It has been definitely established that the cell which is of more rapid growth will often overrun and may finally crowd out altogether the other constituents of the tumor. Thus arise the rhabdomyomas, the chondromas—the simple tumors of the sex glands. In this uncontrolled competition the most embryonal type of cell would have a decided advantage. Therefore Ewing concludes that carcinoma testis is a one-sided teratoma. In the light of the foregoing it is interesting to see to which of these two theories the carcinoma curve lends itself.

According to Chevassu's statistics embryomas occur with greatest frequency from twenty-five to thirty years and seminomas from thirty-five to forty (fig. 2). The writer's review of a larger number of cases coincides with the data of Chevassu, the modes occuring from twenty-three to twenty-nine years in the teratomas and thirty-five to forty-one years in the carcinomas.

Comparing the carcinoma testis curve with that of all cancer (Hoffman[1]), of which the congenital cases are too small a propor-

[1] Mortality from cancer throughout the United States Registration Area. All organs and all parts. 1903–1912. (Hoffman, The Mortality from Cancer.)

	MALE	FEMALE
Until 10	1,170	984
10–24	2,028	1,844
25–34	3,757	7,891
35–44	10,750	26,779
45–54	24,431	46,669
55–64	35,327	52,393
65–74	33,745	43,010
75 and over	18,381	24,601

tion materially to alter the general outline, we see that the former has no resemblance to the latter, for in that case it would have a continuous rise to some time after sixty. In brief, the carcinoma testis curve is the teratoma testis curve with the mode slightly shifted.

Since the histogenesis of carcinoma testis has not been traced from either embryonal or adult tubule cells, it is probable that carcinoma testis is of nontesticular origin, and since there is no reason why misplaced cells should so often be of the same type or occur so frequently with teratomas, unless they are of tera-
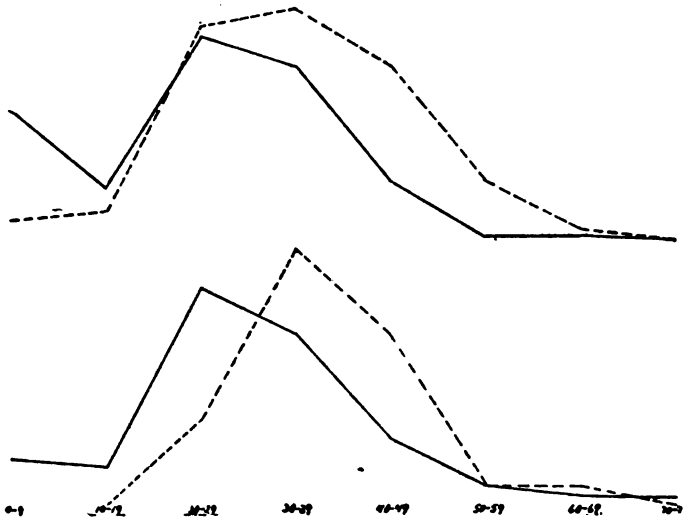
FIG. 2. TESTICULAR TERATOMAS

The lower two curves are reproduced from Chevassu's paper. The continuous line represents the teratomas; the dash line, the carcinomas. In order better to compare the new curves with those of Chevassu, the number of teratomas in each year was divided by four, and that of carcinomas by two. Both curves are plotted on a basis of ten-year periods.

tomatous origin, we are forced to this conclusion towards which the study of the curve gives additional evidence. Similarly to teratoma testis, carcinoma may show a congenital increase in the size of the organ. Morestin (Chevassu) reports such a tumor, which assumed malignancy at the age of thirty-seven. Like-

wise carcinoma occurs more often in undescended testicles. It begins its growth in the rete and, like teratoma testis, is occasionally observed in pseudohermaphrodites.

Ovarian tumors. The origin of primary tumors of the ovary is so undecided that any data in reference to them is of particular interest. Here we shall mention a few facts in regard to one of these tumors which may be of teratomatous origin, i.e., the sarcoma.

Ewing divides these sarcomas into three main types: (1) spindle cell; (2) round cell; (3) myxoma cell. This classification is of special significance since just such types of sarcomatous degeneration of dermoids have been observed (Debucy).

FIG. 3. OVARIAN TERATOMAS

These curves are averages for six year periods drawn to a scale of one-half. The continuous line represents the teratomas with the mode from twenty-three to twenty-nine years; the dash line the carcinomas with the mode from eleven to seventeen years, the period of pubescence.

Desurmont finds that the different primary tumors are bilateral to varying degrees. However, sarcomas, 25 per cent, and dermoids, 20 per cent (Pauli), approximate each other quite closely.

Finally, the most common tumor of infancy is the sarcoma, which is most frequent at fifteen years (Donhauser). Cordier and Zangemeister give fifteen and twenty years, respectively, as the age of most common occurrence of sarcomas. They are found from fifteen years to twenty-five and from forty years to fifty (Desurmont), both periods of physiological stimulation. The writer finds the mode of the combined carcinoma and sarcoma curve at fifteen years (fig. 3). Comparing the mode of this curve with that of ovarian teratomas, we find that it has been shifted forward to the time of pubescence. Hence there is a group of embryonal cell tumors having an age incidence similar to teratomas, and becoming malignant under the stimulation of puberty.

FACTORS IN THE ETIOLOGY OF TERATOMAS

The growth of the earlier teratomas may be adequately explained on the basis of a growth competition between the host and the embryonal rest. But even in the teratomas of infancy another factor, trauma, may be present. It becomes increasingly important later on.

Growth potential. The growth of the host inhibits that of the teratoma.

The growth of the embryonal rest may be divided into two parts: its prenatal development, or growth which continues until stopped by the inhibition produced by the excessive growth of the host; and the growth of which it is still capable (growth potential) after that of the host slows down or ceases. These two parts are in reciprocal relation to each other. The earlier the prenatal inhibition, the smaller and less differentiated will be the inhibited rest but the greater will be the remaining growth potential. Small embryonal rests may develop proliferative powers while large ones, which achieve a certain intrauterine development, seldom if ever show further capacity for spontaneous growth.

Trauma. The shift in the mode of the curve of carcinoma testis from twenty-three to twenty-nine years to thirty-five to forty-one years is due to an external stimulus.

In spite of the larger growth potential of the smaller testicular rests many do not achieve malignancy until their immediate region is traumatized. This is illustrated on comparing these tumors in the testicle and the ovary. A larger proportion of the latter are benign, forming adult structures. The chief difference in their histories is due to their locations; the testicle is exposed to injury, the ovary is not. It is generally admitted that teratomas do not become malignant much oftener than do normal tissues.

In the case of carcinoma testis we should expect with a history of injury to a small undifferentiated rest and the resulting proliferation of an embryonal cell, a shift in the mode of the curve towards that of old age cancer.

SUMMARY AND CONCLUSIONS

1. Growth potential and teratomas

1. Parasite. Teratomas are tridermal embryonal rests endowed with a certain amount of possible growth, i.e., growth potential. When the rest is comparatively large it has necessarily consumed considerable growth energy before birth, while in the small teratoma, the growth period may be divided into two parts, a slight early growth soon followed by an inhibition, and a later, or post-natal growth, should conditions permit.

2. Host. The total number of teratomas in a population, up to any given age, increases while growth potential of the hosts decreases. As the larger increases in the number of teratomas occur in periods when growth of the host is slowed most, the growth of the host must inhibit that of the embryonal rest.

3. The number of teratomas appearing in any given time varies inversely with the growth potential of the host and directly as that of the embryonal rest. The tumors which begin their postnatal growth before that of the host stops are of highest potential, but are not necessarily more malignant, for they must overcome a still present inhibition. Since most teratomas have a low growth potential, they appear most commonly at the time the growth of the host stops—from twenty-three to twenty-nine years.

II. Trauma and autonomous growths

1. Teratomas which start growth as a result of injury are malignant more frequently than those which proliferate solely under the influence of growth potential.

2. When trauma precipitates growth, the teratoma is frequently monodermal. If the inclusion is still in an undifferentiated condition the cell is often of an embryonal type. If a developed inclusion is traumatized the cell in many instances is like that of acquired cancer.

3. The curve of carcinoma testis rises and falls in a manner similar to that of teratoma testis and not like that of old age

cancer. This is another fact which may be adduced in support of the theory that carcinoma testis is a one-sided teratoma. In the female, a similar neoplasm might be expected to arise as the result of the physiologic stimulations of puberty. This is what actually takes place, hence the growth, in all probability, is of teratomatous origin.

4. Trauma is followed by proliferation of cells, and any precipitant of regeneration may be important in the etiology of acquired cancer. In the old the inhibition of the organism is almost negligible. Hence trauma at that time may readily be followed by an uncontrolled and therefore excessive growth. Thus, loss of growth restraint may be almost as important a factor in the etiology of acquired cancer as in that of congenital inclusions.

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[2] These references were chosen out of over one hundred and sixty; they contain extensive reviews of the literature consulted.

THREE UNUSUAL CONNECTIVE TISSUE TUMORS OF THE BREAST

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The comparative rarity of malignant connective tissue tumors of the breast, their peculiar histology, their bizarre gross appearance and doubtful histogenesis seems to justify the presentation here to-night of the following three cases.

CASE I. The first case was on the service of Dr. Hartwell of Bellevue Hospital and the clinical history is as follows:

The patient, 39 years old, first noticed some small lumps in right breast about four years ago. They slowly increased in size and became fairly noticeable about one year ago. Since then the

tumor increased to about ten times the original size. Three weeks previous to admission in hospital the patient suffered excruciating pain in breast and axilla and discomfort from rapidly increasing size of tumor (Fig. 1).

Gross examination reveals a large oval tumor of the right breast, lobulated and cystic in contour, measuring 30 inches in

Fig. 1. Showing enormous size of tumor in Case 1.

circumference, 12 inches in transverse diameter, and 15 inches in long diameter. The skin is not ulcerated or adherent and except for a cyanotic discoloration over the large nodules does not appear involved. The nipple is not retracted but shows a raised edematous area surrounding it. There is no lymphatic involvement.

The left breast shows chronic cystic mastitis, but no neoplasm.

On cross section the tumor reveals firm translucent areas

with many small cysts filled with gelatinous material and a large well-circumscribed hemorrhagic and necrotic portion, which appears surrounded by a definite firm wall suggesting a hemorrhage into a large cyst.

Microscopical section shows a cellular large spindle cell sarcoma with edema and hemorrhage. The remaining areas of breast tissue are transformed into acellular hyaline tissue with a few isolated alveoli and ducts. These are associated with many large dilated blood vessels and multiple hemorrhages.

CASE II. This case occurred at the Memorial Hospital on the service of Dr. Strobell.

The patient, 43 years old, noticed a small tumor in the left breast nine minthe ago, which grew rapidly. The family history was negative.

Gross examination reveals several large and small indurated and irregular masses, in the lower half of right breast and soft swelling in right supra-clavicular region. The breast and tumor measure 21 × 18 cm. deep. There is a fungating mass with central excavations protruding from an opening in the skin 7 cm. in diameter just below the nipple. This opening leads into a large cavity discharging necrotic and putrefying tumor tissue. On cross section there is found a well-circumscribed, encapsulated, soft, translucent tumor, centrally excavated, 18 cm. in diameter. An additional tumor of characteristic papillary fibro-adenomatous type lies just beneath the main tumor. This measures 6 cm. The small remaining portion of the breast shows diffuse fibrosis.

Microscopical examination shows the main tumor is a cellular edematous spindle cell sarcoma arising from fibro-adenoma of intra-canalicular type. The smaller outlying tumor is an intra-canalicular and papillary fibroma. The remaining breast tissue shows fibrosis.

CASE III. The patient, aged 54 years, noticed a small lump in breast twenty-five years ago, which did not increase in size until about two years ago.

Gross examination shows nodular, irregular, circumscribed tumor mass involving the greater part of the right breast. There

is skin involvement and no enlargement of regional lymph nodes. On cross section one finds firm dense nodules in whorl arrangement occupying the greater part of the tumor, with a few small hemorrhagic areas. In the lower outer portion of the breast there is a well-circumscribed hemorrhage 1 × 1 inch with well-defined wall.

Microscopically the main mass of the tumor consists of small spindle cells arranged in whorls and interlaced bands, typical of a fibro-sarcoma. The remaining breast tissue at the margin of the tumor shows extreme fibrosis with hyaline degeneration which surrounds the ducts and atrophied alveoli.

In a study of these cases several facts demand particular attention: First, the relatively long quiescence of small nodules in the breast. (Gross reports a case in which a nodule remained of the same size for 36 years and at the age of 63 began rapidly to grow, doubling its size in one year. Robin reported a similar case.) Second, the sudden and extremely rapid growth at a later period. The age incidence of spindle cell sarcomas varies, usually, from 39 to 49 years. The increase in size is not only more rapid than in other tumors, but varies widely and is usually influenced by their structure, the presence or absence of cysts or associated hemorrhage. This is well shown in the first case reported where the enormous size was in great part due to the large hemorrhages into cysts. Third, ulceration of the overlying tissues is rather common, occurring in about 18 per cent. of the reported cases. Ulceration occurred in the second case reported here and showed the characteristic type of deep fungating ulcers. The histogenesis is the point of especial interest in these neoplasms and opens a wide field for speculation. Undoubtedly in the majority of cases a previous intra-canalicular fibroma proves the starting point of these neoplasms, as is well shown in the second case from the Memorial Hospital. However, Case No. 1 shows no evidence of a previous benign neoplasm and was undoubtedly preceded by an extensive diffuse fibrosis of the breast, as evidenced in the remaining marked diffuse fibrous mastitis which is observed in the other portions of the mammary gland and was also noted in the opposite breast. The short duration in this case

is against the theory of a preceding benign fibroma.   It is not un-
reasonable to suppose that a diffuse fibrosis may precede this type
of cystic sarcoma.   In the third case we have neither a fibroma
nor marked extensive fibrosis to suggest a point of origin.   The
histological structure with the arrangement of spindle cells in
whorls and strands so often encountered in fibro-sarcomas of
neurogenic origin leads one to suspect an origin from nerve
sheaths of the same histological character and histogenesis as the
neurogenic sarcomata of other regions.

Discussion:

DR. EWING: We have removed early this week at the Memorial Hospital
another specimen of sarcoma of the breast, about two thirds as large as the
largest specimen here.   It occurred in a woman sixty-five years of age.   It
had existed for several years, but had grown rapidly during the past six
months.   The patient was decidedly emaciated and cachectic through absorp-
tion from several softened necrotic areas in the huge tumor mass.   The skin
was intact, and the tumor well encapsulated.

THE PLACE OF PATHOLOGICAL ANATOMY IN MILITARY MEDICINE

By JAMES EWING

Contract Surgeon, Army Medical Museum

(With one illustration)

THE essential importance of pathological anatomy as a basis for medical and surgical practice and research is everywhere conceded, but probably nowhere fully heeded. Although medicine is distinguished from the medical cults by nothing so much as by the reliance placed upon the anatomical lesions of disease, yet an accurate knowledge of what disease does in the body is certainly not possessed by the majority of regular practitioners. Even in the most modern medical schools the curriculum does not permit the student to become familiar with the innumerable gross lesions of disease, and the material available would not permit it if the curriculum did. At a recent examination held by the National Board it transpired that none of the candidates and few of the examiners had ever seen the liver of eclampsia, although hemorrhagic hepatitis is one of the most striking and significant gross lesions occurring in the human body.

In the modern highly equipped university clinic it is commonly assumed that the chief has, of course, acquired competence in such fundamental matters as pathological anatomy, but the record does not always show how he acquired this knowledge, and investigation often reveals that this branch of the work of the clinic is in charge of some young assistant who is not always marked for rapid advancement. .

Even among pathologists there is a wide divergence in the extent to which pathological anatomy is cultivated, as a basis for teaching pathology or as a means of research. Very often it is assumed that gross pathology is such an old subject that the cream has been skimmed from it and that no important additions to our knowledge are to be expected from it, and certainly no sensational rewards.

Chief among the reasons for the present decline of pathological anatomy in America is the difficulty of securing material for study. The proper performance of an autopsy is a difficult, laborious, expert and time-consuming procedure. It involves a peculiar knowledge of normal anatomy, technical skill, a systematic and yet adaptable procedure, attention to microscopic, bacteriologic, and chemical data, wide acquaintance with the lesions of disease, broad

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knowledge of medicine, and acquaintance with technical language. The opportunity to perform autopsies is hedged about by an unintelligent attitude of the public, by antiquated laws, by inadequate facilities, and by the conflicting interests of anatomists, undertakers ecclesiastics, burial societies, and other friends. In New York City it is a misdemeanor to remove organs from the body in municipal hospitals, and thus properly to teach pathological anatomy.

To the believer in the renaissance of pathological anatomy two hopeful signs appear on the horizon. One is the approach of state medicine, and the other is the demand for military medical efficiency. The moment that the sponsors of either of these movements begin to analyze the elements of real efficiency in the knowledge and practice of medicine, that moment a high development of pathological anatomy is assured.

The reason why pathological anatomy has had such a fundamental influence on the progress of medicine and is so essential to the practitioner lies in the fact that tissue changes detected by the naked eye had interpreted by microscopic control are just those that produce the significant symptoms of disease and, as a rule, determine the main events in its course. It is only upon a sound basis of pathological anatomy that the bacteriologist, chemist, and serologist can safely proceed to trace the finer factors in the causation and pathogenesis of disease. It is a matter of historical record that while it has been the task of the clinician to point out the clinical problems of medicine, it was first the gross pathologist who found how these problems were to be attacked. Thus when Virchow, seventy years ago, pointed out that in uremia, lesions of the liver were quite as prominent as those of the kidney, he placed the problem of Bright's disease on the doorstep of the physiological chemist. For many years before attention centered on the pancreas in diabetes, pathological anatomists had emphasized the nearly constant occurrence of lesions in this organ, and only recently has the simple morphological study of the organ established the importance of lesions in the islands, and justified the pursuit of the pancreatic hormone. Even with the infectious diseases it is a rule that a specific microörganism, producing specific clinical symptoms, causes also characteristic gross anatomical lesions which in turn present a peculiar histological structure, and while, with some exceptions, the fever and intoxication are of general origin and of non-specific character, the grosser anatomical lesions determine the main clinical features of the disease. Some may not concede such central importance to pathological anatomy, but I

venture to think that the above estimate is not overdrawn, and could be supported by very numerous additional data drawn from all departments of medicine.

The importance of pathological anatomy in the Army Medical Service has been plainly illustrated in many ways.

When the demands of the war suddenly developed and it became necessary to equip a large number of laboratories with a competent personnel, it was soon apparent that American laboratories had been manned chiefly by clinical pathologists, while the supply of expert pathologists was quite inadequate.

When a large new Army Medical School was established at Fort Oglethorpe, the effort to secure pathological material for teaching purposes soon revealed that there was no excess material of this sort, and that it was difficult to secure.

When epidemics of pneumonia and meningitis broke out in cantonments the best opinion was unable to decide whether the disease was exhibiting new or only old features, because there was no opportunity to compare the lesions on a large scale with those previously observed. Neither clinicians nor pathologists of today remember the details of the influenzal lesions of 1889-90, and nowhere have these lesions been properly preserved. If there is, in America, any collection of post-measles pneumonias, or any physician thoroughly acquainted with the details of such lesions, it was not apparent in the exigency of last winter.

When the treatment of infected war wounds became the most urgent surgical problem, the solution was reached on the basis of the grossest of pathological anatomical grounds, namely, the excision of wide areas of devitalized muscle. It is now apparent that a few careful dissections of these wounds would have revealed to the naked eye the extent of the devitalized tissues, the dissemination of bone fragments, and the real problem of surgical treatment. The question of when to amputate or save infected fractured limbs seems to have been influenced strongly, at least in British circles, by the amount of damage to heart and kidneys revealed at autopsy on prolonged cases of suppuration or after less stormy wound healing.

Such considerations as the above early led our British confreres to recognize the importance of studies in gross pathology in the elucidation of military medical problems, and to appoint a committee of experts to develop the field of pathological anatomy and museum demonstration. An impressive exhibition of some of their results was recently held in London.

Likewise our own Medical Staff, with somewhat less definite evidence of its necessity, and with correspondingly greater foresight, many months ago formed plans to collect all the pathological material from the army hospitals, at home and overseas, and to form a new Army Medical Museum at Washington. The objects of the collection were conceived to be:

1. The gathering of the objective records of the effects of disease, from which might be written an important chapter in the medical history of the war.

2. The employment of pathological anatomical studies in the solution of current military medical problems.

3. The formation of a permanent museum.

4. The use of the material for the education of military physicians in the diseases occurring in the Army.

5. The later study, both gross and microscopic, of large numbers of cases, for their general scientific and special military interest.

At the present time it seems desirable that those who are interested in the undertaking should be apprised of the degree of success thus far attained, of the general objects, methods, and possibilities of the project, so that during the remaining period of post-bellum activities every effort may be made to bring the project to complete success.

It is necessary to admit that thus far, while much valuable material has been collected, and much more is stored in hospital laboratories, the indications are not entirely reassuring that the American Army Medical Staff will have in its possession at the end of the war period a satisfactory collection of pathological material from which to write the medical history of the war, and it appears even less certain that this material will permit the formation of a museum which is abreast of modern standards. A frank acknowledgment of this situation seems to be its most likely remedy.

Prominent among the causes of this partial failure has been the great haste demanded in the military preparations, which had to be the first consideration. The outbreaks of infectious diseases in the camps, although probably not more severe than with some of our allies, threw a vast amount of emergency work upon the laboratory staff, and made it impossible to attend to the many details of preparation and collection of pathological specimens. Even more important was the paucity of men trained in the methods of the pathological laboratory, as distinguished from the clinical laboratory, a defect that must be charged against the present organization of American civil medicine. The war definitely demonstrated

that there is shortage of pathologists and pathological facilities in America, while clinical laboratory diagnosis is rather highly developed.

Finally, while there is in America an association composed of rather numerous pathologists interested in museum work, their standards and propaganda have not yet penetrated widely, so that the prevailing notions, at least among the clinical pathologists, of what constitutes a museum specimen are quite vague. Even among pathologists, in and out of the Army, there is a divergence in ideas and practice regarding museum preparations. The spirited inquiry has been made whether an autopsy is performed to settle important questions at the autopsy table, or only to secure museum specimens. On this point it is of first importance to come to an understanding. The writer has no hesitation in affirming that both the above objects should be considered in performing an autopsy, but that the destruction of a valuable museum specimen for the sake of a hasty demonstration, especially in the case of an American soldier, should be deprecated. If the Army Museum is to take a creditable form, the army pathologist must resolutely refuse to damage gross anatomical specimens, but, on the contrary, must use all his ingenuity to preserve in natural form and color a reasonable or even a large proportion of the lesions that come under his hands.

The importance of collecting museum material being recognized and the means provided, the question remains: How extensive shall this collection be, and what methods shall be employed in gathering it?

Those who conceived the plan of creating a new pathological museum believe that all material obtained in the army service should be sent to Washington, and orders to that effect were given out some months ago. If these orders are rigidly followed, it appears certain that much material of little or no value will accumulate. Yet the responsibility of throwing specimens away can best be assumed by a central office. A few tonsils in a camp laboratory may be of little interest, but a thousand gathered from many sources would serve to determine what significance the tonsil has in military service.

The same rule applies to all the specialties. The histopathology of cutaneous diseases may be a threadbare subject, but the Army needs such a collection for several purposes. Or it may prove that there are still some new facts to be elicited by the study of lesions of the skin, especially those due to poisonous gases and burns.

In the case of general diseases the scope of the material which

it is desirable to include in the museum collection will vary in the opinions of different pathologists. Yet if any competent and systematic study of diseases such as pneumonia, measles, and influenza, is to be undertaken, portions of all the organs, and not merely the main lesion, must be preserved for histological examination. Exactly the same rule would seem to apply in the preservation of gross material for museum demonstration. The idea of exhibiting in natural form and color all the affected organs in a series of cases of the important diseases occurring in the Army may appear unreasonable or even grotesque. It is a fact that it has never been accomplished. Nevertheless the writer believes that the time has come when the full possibilities of museum demonstration—for record, for education, and for research—should be recognized, and a strong effort made to realize them in the new Army Medical Museum. Such an undertaking would call for new standards in gross pathology, but the war has brought new standards in nearly every other branch of medicine, and why should not pathological anatomy be allowed to take a step forward?

The pathologist is commonly content to save a portion of one lung in pneumonia, put it in a glass jar, and use it as a text on which to discuss the pathogenesis of the disease. The extent of the pulmonary, bronchial, and pleural lesions, and the highly important changes in the other organs are left to the imagination. Yet it is just these peculiar associated lesions, so difficult to describe and correlate, that render the picture significant and impressive. On the old basis, pathological museums, interesting enough in themselves, have never assumed the importance they deserve, and have never fully discharged their functions.

No better proof of the necessity of presenting the whole picture of the disease could be found than has just appeared in the recent epidemic of influenza. The urgent question arose whether the disease was new or had been seen before. This question, not solvable on the available bacteriological evidence alone, could have been decided by a close comparison of the peculiar lesions occurring in the lungs and other organs in October, 1918, with those observed in the winter of 1917-18. As observed in October, these peculiar features included the hemorrhagic lesions in the lungs and respiratory tract, the hemorrhagic gastritis and enteritis, and the alterations in the hemopoietic organs, all of which were required if the picture were to be in any sense adequate and conclusive.

On this account the following scheme of procedure was prepared at the Army Medical Museum and recommended to a few neighbor-

ing hospitals. It is presented here, not only for its immediate rela-
tion to influenza, but as representing the object to be attained by
the museum pathologist in dealing with other diseases. That object
should be to preserve the whole picture of the disease.

ARMY MEDICAL MUSEUM

PROCEDURE RECOMMENDED FOR THE PRESERVATION OF MUSEUM MATERIAL
IN CASES OF INFLUENZA

Save the Whole Picture of the Disease.
Use Klotz's fluid, Kaiserling's, or 10 per cent formalin.
Respiratory organs.—Remove both lungs together, and with them, if
possible, the tonsils, larynx, and trachea.
Make bacterial cultures from posterior surfaces of lobes.
Gently distend the lungs to normal dimensions by pouring fixing fluid
into trachea. Wait one or, better, twenty-four hours before incising.
Lay out lungs on their anterior surfaces, and with a long knife bisect the
bronchial tree to the second bifurcations, continuing the incision and remov-
ing in the same plane one to two inches of the posterior portions of the
parenchyma, from apex to base.
Save the smaller portions for histological study, and float the other in a
large container.
Stomach.—Half fill the stomach with fixative, float in a large container,
and after 24 hours incise along middle of anterior surface. Treat similarly
affected portions of small and large intestine.
Spleen.—Bisect in long diameters.
Liver.—Preserve a transverse segment 2 inches thick.
Kidneys.—Bisect with adrenals and preserve one half.
Bone marrow.—Remove a portion of sternum and body of one vertebra.
The head of the humerus in adults does not usually contain red marrow.

Objections to the prominence thus given to pathological anat-
omy will arise from the requirements of the bacteriologist, from
the considerable demands on the time of the laboratory staff, and
from the respect due to the dead body.

Yet the bacteriologist need not cut the lung to pieces to obtain
cultures. He can make sterile punctures at as many points as de-
sired. In fact it seems not impossible that the complex bacteriology
of the pneumonic lung might be simplified by coöperation between
pathologist and bacteriologist, identifying and isolating the
numerous secondary lesions and infections that occur, and possibly
by assigning different organs to different purposes.

The labor of preparing organs for gross demonstration is con-
siderable, and special experience and skill are required, so that it
would seem desirable to provide a special laboratory assistant part
of whose time should be devoted to this work.

As a rule, no unusual procedures are involved at an autopsy performed with full attention to museum requirements.

As with pneumonia, so with other diseases, the objects of an autopsy should include the preservation of the whole picture of the disease. In not a few instances this result has already been attained by army pathologists, who have sent to the museum very complete series of lesions illustrating certain maladies. Thus, in a case of malignant endocarditis, the organs include the heart with ulcerated valves, lungs with broncho-pneumonia, spleen and liver with infarcts, and kidneys and intestine with petechial hemorrhages.

In a group of cases the lesion extends by continuity through many organs, and its demonstration requires an elaborate dissection. The museum staff is prepared to make such dissections. In a case of thymic tumor involving mediastinum, lungs, liver, mesentery and peri-renal tissues, the organs were received *en masse*, and by a laborious dissection the anatomy of this condition is displayed in a highly interesting and instructive manner. In a case of Addison's disease the abdominal organs were received *en masse*, and a minute dissection of these organs, with lymphatics, sympathetic system, and adrenals, reveals in a most impressive way the distribution of a caseous tuberculous lesion. This specimen is a unique contribution to the anatomy and pathogenesis of Addison's disease.

In certain cases the material to be saved will be suggested by the pathologist's knowledge of research problems of current interest. Diseases of the endocrine system suggest attention to all the organs of this system. The anatomical signs of status lymphaticus are of very general distribution. No one would fail to examine representative portions of bone marrow, chains of lymph nodes, and the several tissue lesions in leukemia.

Attention may be drawn here to the importance of studying the anatomical changes in any limbs amputated in reconstruction hospitals, since it appears probable that the knowledge thus gained may guide the orthopedist in devising operations and apparatus for the treatment of these cases.

Today it is not sufficient, as it was in Civil War time, to illustrate infected fractures by means of a dry bone specimen, for equal or greater interest attaches to the changes in the soft parts and, in fatal cases, to such complications as endocarditis, fat embolism, and nephritis.

Regarding the methods to be employed in the preservation of museum and histological material, reference may be made to the

LESION OF RESPIRATORY TRACT IN INFLUENZAL PNEUMONIA

Circular of Information sent to army hospitals last September.
The essential factor which makes it possible for the modern
pathological museum to present the exact condition of diseased
organs is the blood-preserving property of formalin. When the
blood is hemolyzed no method as yet devised gives satisfactory
preservation of color. One who knows how to use formalin can
obtain almost as good results with a 10 per cent solution as with
any of the more complex fixatives, but the care required is some-
what greater than with Klotz's or Kaiserling's fluids. Gross speci-
mens should not remain in formalin more than two or three days,
they should then be washed in running water, color restored in alco-
hol for 24 to 48 hours, and preserved in the final fluids recommended
by Klotz or Kaiserling, paraffin oil, or sugar solution. The great
advantage of Klotz's fixative, No. 1, is the fact that specimens may
remain and be shipped in it. As with most other technical prob-
lems, the skill, patience, and ingenuity of the pathologist will
always be taxed to secure desired results.

Although the war is over, there will still be abundant oppor-
tunity for the army laboratories to employ the best methods and
aim at the highest standards of pathological anatomical prepar-
ation. In fact, as the stress of military activity lessens, the situa-
tion becomes more favorable for attention to the interests of
museum collection and the study of medical problems arising from
the war.

It may be emphasized that the Army Medical Museum is
equipped to make the best use of an extensive museum collection.
With a staff of experts in plaster and wax modelling, painting,
photography, and anatomical preparation, it is capable of employ-
ing every branch of pictorial art on the material received, and thus
to become a powerful factor in medical education. In the remain-
ing period of military mobilization, a concerted effort should be
made to gather at Washington a complete and modern pathological
collection. If such a result can be accomplished, it will give a
powerful impetus to the study of pathology and distinctly elevate
the standards of medical education and practice, both in the Army
and throughout the country.

PULMONARY LESIONS OF INFLUENZA PNEUMONIA, AND A METHOD OF MUSEUM PREPARATION

JAMES EWING, M.D.

My object in presenting these slides this evening is to demonstrate a method of preparation of lungs of influenza or other pulmonary diseases for gross anatomical study, and for permanent preservation in a museum. We might ask perhaps what is the use of making a separate topic of a matter of this sort. I found this summer at the Army Medical Museum at Washington that there was very great need for information on this subject, and it was only after a considerable and harrassing experience that we were able to obtain any satisfactory gross material, that is, which demonstrated the lesions of influenza in a fully adequate manner. It was only after a number of trials and experiments that we adopted a method which will be demonstrated in these slides. That method consists briefly in attempting to demonstrate fully the lesions in both lungs, because without the demonstration of the lesions in both lungs one might get a very erroneous idea of the nature of the pulmonary lesion in influenza. I had many consultations with Dr. MacCallum, and it is owing to his energy

that we have any material at all from the epidemic of so-called
post-measles pneumonia in 1917.   Knowing that the lesion is
bilateral, and may vary considerably in both lungs, we agreed that
we must have both of them for study. Finally, at St. Elizabeth's
Hospital, we perfected a method which is easy to describe, but
not so easily applied.   The plan is to lay the lungs, removed
together, on their anterior surfaces and to cut both lungs down
the bronchial tree.   With a long sharp knife make a slice where
the bronchial tree is running.   That cuts off the posterior portion
of the lung a slice about two inches thick from the apex to base.
The first cut may not strike the bronchial tree throughout its
entire length, but one can make another cut which will continue
the section to the finest ramifications of the bronchial tree, and
when this is done, it gives a striking picture (Fig. 1).   There were
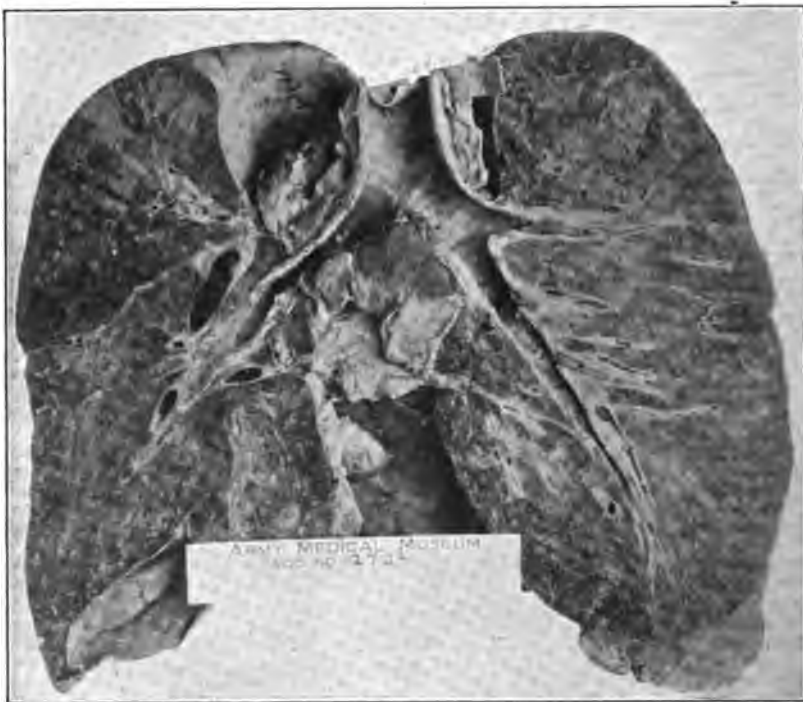
FIG. 1.   A characteristic type of bronchopneumonia following influenza.

several results of the study of lungs sectioned in this way which I think would not have been secured by cutting the lungs in a haphazard manner, as is usually done.

In studying the lungs we found what I call a critical area around the centers of the main bronchi in both lungs where as a rule secondary pneumonic processes are apt to begin. I had quite a discussion with some internists once on the question whether pneumonia began under the pleura or in the central area, and I could not answer the question. In our own museum specimens there were illustrations of both conditions. In a series of sections cut in this way through both lungs we have found in a large proportion of post-influenzal pneumonias that consolidation began in an area around the bronchial tree, which became so common that we designated it as the critical area. Another point which has been brought out, and which I think could only have been done to full satisfaction by this method of section, was that in practically all our cases the original lesion was a bronchiolitis. Sometimes a secondary and diffuse pneumonia would start all through a lobe or through several lobes, so that on inspection one could not make out a trace of the original bronchiolitis, but in a high proportion of cases one would find a tell-tale peripheral zone of fine bronchiolitis. Another matter which was emphasized only lately, and which can be demonstrated in the slides, was the characteristic condition of the lung in most cases of empyema. I had never seen it before. I have been told that Ribbert described it, and also have been told by members of the Empyema Commission that they had concluded that a peculiar type of lesion must exist in the lung in cases of empyema. Here a streptococcus infection spreads through the lymphatics of the bronchioles and of the septa and thus reaches the pleura. It passes rapidly through these lymphatic channels, and distends them with pus to a very noticeable extent. In the earlier stages of this type of lung one finds the septa clearly marked out in a characteristic mosaic. This lesion might almost be spoken of as an abscess, but it is not a true abscess. There is no liquefaction, but the lymphatics are very widely distended with exudate. This lesion, occurring in

one or both lungs, can be satisfactorily traced to the root of the lung only in sections made in this particular manner.

I do not expect this evening to give a complete demonstration of the pulmonary lesions in influenza, but hope only to show the main features, and I trust that the slides will impress on the members of the society how important it is for pathologists to take pains in preserving specimens.

LANTERN SLIDE DEMONSTRATION

The slides were arranged in the following groups:

1. Diffuse bilateral hemorrhagic pneumonias. Bronchiolitis obscured by blood. Cases from height of epidemic.

2. Fine bronchiolitis all over both lungs, which are very red, but show little exudate.

3. Lobular or diffuse pneumonias obliterating the bronchiolitis except at periphery.

4. Increasing prominence of bronchiolitis and bronchopneumonia, with or without patchy pneumonias.

5. Necrotic bronchopneumonias resembling tubercle.

6. Lungs in empyema.

7. Comparison of lungs of 1918 with those of 1917.

One of the conclusions resulting from a comparison of the lesions of 1917 and 1918 caused much opposition, and of course it was based only upon such evidence as pathologico-anatomical data. But I think that the fact that from the gross anatomical standpoint the lungs of the so-called post-measles pneumonia of 1917 correspond in even minute detail, with the lesions that have been found in the epidemic of post-influenza pneumonia, is suggestive that these pneumonias are essentially the same. This fact hardly permits one to state such a conclusion as final. The nature of these diseases is to be decided finally by bacteriological data, but since the bacteriologists have not as yet arrived at any conclusion in the matter, it seems to me it would be wise not to dismiss entirely the evidence which may be obtained from the gross anatomical study of the lungs. It may also be

noted that in 1917 influenza bacilli were frequently found and reported, but camp bacteriologists considered them of no importance. This was not noted until the reports were reëxamined in 1918. I think it is desirable to emphasize the wisdom and necessity of making every effort to preserve the characteristic specimens of these lesions. The question arises what is the relation of post-measles pneumonia to pure measles. It is not easy to answer this question. Few if any museums possess a well-preserved set of lungs in a case of measles. I think it would be very important evidence if we could point out that this or that particular specimen belongs to measles. It has been my hope that we could get a group of selected specimens which would show every variation in the type of these influenza and measles pneumonias which might settle some of these questions from the gross anatomical standpoint and form a basis for the study of the etiology of these diseases on the bacteriological side.

Discussion:

Dr. Cole: I will not discuss this paper more than to express my appreciation of the splendid work Dr. Ewing has done in studying the pathological lesions in these cases of pneumonia. It is not only in bronchopneumonia that our knowledge is lacking of the exact pathological lesion. We have been interested for a long time, more from the bacteriological standpoint, in lobar pneumonia, and I have been struck with the lack of exact knowledge of the lesions, and I think a careful study of the lungs and the lesions produced in lobar pneumonia would give us a good deal of valuable information concerning the nature of this disease. In regard to the lungs described to-night by Dr. Ewing, one is struck by the great variety in the lesions. It is quite true, as Dr. Ewing states, that certain of these lungs show lesions which are like those we saw in 1917 and again in 1918, before the present epidemic of influenza occurred. I studied the pneumonia occurring at San Antonio in the winter of 1917–18. It is true we found the influenza bacillus in the lungs in many of the cases, but we did not lay stress upon this. Most of the cases of pneumonia with peculiar lesions occurred in association with measles, but we pointed out that similar cases occurred without measles. If the disease we were studying was influenza, it is difficult to understand why it should have taken on the remarkable epidemic character which it has exhibited this winter. I do not know what influenza is, except that it is an acute respiratory disease occurring in remarkable epidemic form and usually associated with leucopenia. In going over the records of our cases during the epidemic I find all clinical varieties of respiratory disease which we had to label " influenza,"

from coryza to the most severe bronchopneumonia, and it is impossible to say from the clinical features alone that "this is influenza" and "this is not influenza." One point I should like to inquire about is the peribronchial infiltration, which was such a marked feature in the measles cases. Many of these cases associated with empyema showed also a severe lymphangitis with dilatation of the lymph vessels running out to the periphery. Some of the cases showed marked bloody exudate and extreme edema of the lungs, as have been seen in the present epidemic. Such cases are still occurring. Only the other day we had such a case and the same day another case with staphylococcus infection, the lung showing numerous abscesses. In both of these cases influenza bacilli were isolated from the lung, and it seems that both cases must be considered as associated with the epidemic through which we have just passed. None of the lung lesions so far described seem to me definite enough to permit us to consider them specific. With the clinical picture so variable and with no well established etiology I still have difficulty in defining influenza.

DR. MANGES: Does Dr. Ewing adhere to the classification that Dr. MacCallum has so recently introduced, that is, that the influenza bacillus gives a typical picture, as does the streptococcus and the staphylococcus? I judge from the pictures this evening that they are quite uniform types. I should like to ask Dr. Cole whether in the measles cases there was a typical infiltration of the lungs. I think we have to add to the leucopenia a slow pulse and a slow respiration, which we must include in the medical picture of influenza. I think those of us clinicians who are here to-night can only appreciate deeply what Dr. Ewing has given us in his presentation. It emphasizes more than ever the extension of the disease from the so-called critical area. Another point is that of the dilated bronchi in relation to empyema cases, and that is one which is being overlooked constantly, and which would also explain the gravity of the case in empyema, which is also often overlooked.

DR. WILLIAMS: The evidence that Dr. Ewing gives of post-measles lungs being similar to the so-called influenza lungs adds somewhat to the evidence in favor of the influenza bacillus being a secondary invader in the pandemic.

DR. MOSCHCOWITZ: Dr. Ewing's description of the erysipelas of the lung interested me especially. My brother, Major A. V. Moschcowitz, who is a member of the Empyema Commission, tells me that invariably in the cases of empyema there is a subpleural abscess that has ruptured. I would like to ask Dr. Ewing if he has found that to be the case.

DR. EWING: I should like to emphasize the fact that while I am impressing the importance of pathological anatomy, I do not wish to overemphasize it. It happened to be my job to do something in this field, and I went to it with the unsual energy which we would all display in such a case. I have not said anything about the histology of these lesions, although I can assure you it is just as variable as the gross anatomy, but in general I think there is one central tendency which is quite striking. Throughout the entire series of post-measles and post-influenza pneumonias, usually complicated by streptococcus infection, whenever there is a bloody exudate and a severe distortion of the walls of the bronchioles, there is a certain tendency toward

organization. In a great many of the earlier cases organization was not found, but it was extremely common in the later specimens.

As to the bacteriology of these lungs, quite a corps of assistants worked in attempting to trace the bacteria demonstrable in sections of the lungs. It is a very laborious process, and one about which you do not see very much said in the reports of the laboratories. I think in general there is a very wide variation in what one will find using Gram's stain, carbol-fuchsin, etc., in studying these lungs. Some lungs show no bacteria, that is, in the portions which come in the section. Others show a tremendous overgrowth of staphylococcus, pneumococcus or streptococcus. There are all variations. In not a few one can find a few Gram-negatives, along with an overgrowth of Gram-positives, leading to the conclusion that it is the Gram-negative organisms which sweep over first, then disappear, and are replaced by the Gram-positive types.

In regard to Dr. Cole's question of the round celled and perivascular infiltration that is present, I understand that every time one says " broncho-pneumonia " he means a lesion affecting the walls of the bronchioles and the walls of the air vesicles immediately surrounding them, and that this infiltration is essentially an interstitial process. The infiltration consists of round cells, lymphocytes, and plasma cells. The process is essentially a productive inflammation. I think Delafield interpreted it correctly. In addition to the interstitial inflammation, there is nearly always an acute purulent catarrhal exudate into the lumina of the bronchioles, and that is present in practically every one of the lungs studied. The point Dr. Cole makes as to the existence of an essential and specific anatomical lesion in influenza seems to me is the essential point in the whole discussion. If we could establish that there is an essential lesion in influenza-pneumonia then we could go a long way toward determining the relation of these different types and form the basis for the bacteriological study. Now there appear to be sufficient data on which to construct a specific lesion for the lungs in influenza. I think it consists of a purulent bronchiolitis with bronchopneumonia limited to the tissue immediately around the bronchioles, uniform, and involving the finest ramifications of the bronchioles. That I think is pretty nearly a specific thing from the anatomical standpoint. We may not be able to recognize the condition clinically. But its bacteriology has not been settled yet, although from some sources it is said that if you get an early lesion you will find the influenza bacillus in practically pure culture in the pus from the bronchioles. I think all this points to the conclusion that the lesion which I have described is the specific thing in what you call influenza. The fact that this lesion often occurs in measles does not indicate that measles and influenza are the same disease, but merely suggests that such post-measles pneumonias are influenza grafted on measles. In regard to abscesses in the lungs, I heard that statement from several sources, and can only partly confirm it. Subpleural pus is present in practically all cases of empyema, but it is a lesion which I would not call an abscess at all. What you see is a phlegmonous lymphangitis, spreading so widely as to resemble a phlegmonous erysipelas, and not an abscess. The lung tissue is not destroyed; it is widely spread apart by pus and edema, but there is no destruction of the tissue.

DIFFUSE ENDOTHELIOMA OF BONE

JAMES EWING, M.D.

For some years I have been encountering in material curetted from bone tumors a structure which differed markedly from that of osteogenic sarcoma, was not identical with any known form of myeloma, and which had to be designated by the vague term "round cell sarcoma" of unknown origin and nature. I had no opportunity of following the course or learning the outcome of these cases, as most of them were treated by amputation of the limb.

Recently a case came under observation at the Memorial Hospital which revealed that this tumor is highly susceptible to radium, a fact that convinced me that the disease was entirely different from osteogenic sarcoma, which resists treatment by the physical agents.

The story of this case is briefly as follows:

A fourteen-year-old girl had been treated by an outside physician in 1918 for nasal discharge and occasional bleeding. Some ocular symptoms led to the suggestion of congenital lues, and a Wassermann reaction being weakly positive, salvarsan was administered. In November, 1918, while pulling on a rope, a spontaneous fracture of the ulna occurred, followed by swelling which gradually subsided. In January, 1919, the swelling recurred and continued with pain and disability until a well-marked tumor occupied the upper part of the arm. This tumor was noted to fluctuate in size. The veins of the skin were dilated, and the appearance led to the diagnosis of osteogenic sarcoma. Eight injections of Coley's toxins were administered at Mount Sinai Hospital, without notable effect.

On April twelfth at the Memorial Hospital a radium pack of 12,760 millicurie hours was applied to the arm, and followed by two other packs at intervals of two weeks. The tumor began to recede at once and at the end of five weeks no external swelling remained.

On admission the radiograph showed a peculiar diffuse fading of the upper half of the shaft of the radius, and a faint line from the old fracture. The outline of the slightly swollen shaft was smooth (Fig. 1); there was no

bone formation, no point of perforation, or area of erosion of the shaft, all of which features told against osteogenic sarcoma. The prompt recession

FIG. 1. Diffuse endothelioma of radius. Diffuse absorption of shaft; spontaneous fracture; invasion of soft parts.

under radium was also quite unlike our experience with osteogenic sarcoma (Fig. 2). With the recession of the tumor the shaft was well restored and normal function regained. The patient left the hospital with instructions to return weekly for observation, which was continued for several months.

FIG. 2. Diffuse endothelioma of radius. After radium treatment.

The patient then came under the care of her original physician who noted persistence of the nasal and ocular symptoms, and, regarding the tumor of the radius as luetic, he instituted vigorous treatment by salvarsan. The injections, however, were followed by severe toxic symptoms, vomiting, bloody urine, collapse, and progressive anemia. Later injections of cacodylate of sodium were administered for the anemia. The patient failed steadily and the tumor of the arm began to reappear. There was now an

irregular fever up to 103° F. The urine failed to show Bence-Jones protein.

In October, 1920, the patient returned to the Memorial Hospital with a definite recurrence of the tumor, and owing to the conflict of opinion, a portion of tissue was removed for diagnosis. It proved to be a round cell growth of the above-mentioned type. Other tumors had now appeared plainly in the skull. There was exophthalmos. The eye grounds showed choked disc and nerve atrophy. The radiograph of the lungs was negative. Anemia and cachexia progressed rapidly, and death occurred on December 23, 1920. The total duration was about thirty months.

During the past four months I have seen six other cases of this disease. They occurred in subjects from fourteen to nineteen years of age. The bones affected were tibia, ulna, ischium, parietal and scapula. The tumors grew rather slowly, requiring some months to attract attention, but they were accompanied by attacks of pain and disability. One boy complained only of intermittent attacks of pain after exercise during the summer, but in November a smooth swelling appeared over the upper half of the leg. Several tumors were found to fluctuate in size, a symptom due to their vascularity. All were rather painful and tender.

The radiographs give characteristic features on which a diagnosis may be based with considerable certainty. A large portion or the whole of the shaft is involved, but the ends are generally spared, contrary to the rule with osteogenic sarcoma. The shaft is slightly widened, but the main alteration is a gradual diffuse fading of the bone structure. Bone production has been entirely absent. Some of the bones appeared honeycombed. Perforation of the shaft and sharp limitation of the process are wanting. The central excavation with widened bony capsule, as seen in benign giant cell tumors, is missing. The radiograph is therefore rather specific.

Under radium treatment the tumor recedes and the shaft gradually becomes well defined with little deformity and no eccentric bone formation.

In seven cases the tissue was examined microscopically, and in all the structure was nearly identical. The growth was composed of broad sheets of small polyhedral cells with pale cytoplasm, small hyperchromatic nuclei, well-defined cell borders, and

complete absence of intercellular material.   Hydropic degenera-
tion often affects large islands of cells, in which only nuclei and
cell borders are visible.   Necrosis occurred after radium appli-
cations.   There is very little desmoplastic quality, but the tumor
cells readily infiltrate muscle and pass along the fasciæ.   In none
were pulmonary or other forms of metastases observed.   In the
case cited the tumors of the skull were regarded as primary and
of long standing.   In some sections the cells were of increased
size, while in others they were smaller and more compact, and
approached the morphology of plasma cells.   However, no defi-
nite areas of plasma cells have been seen in any case.

The probable endothelial nature of the tumor was suggested
by the form of the cells, and especially by the appearance in broad
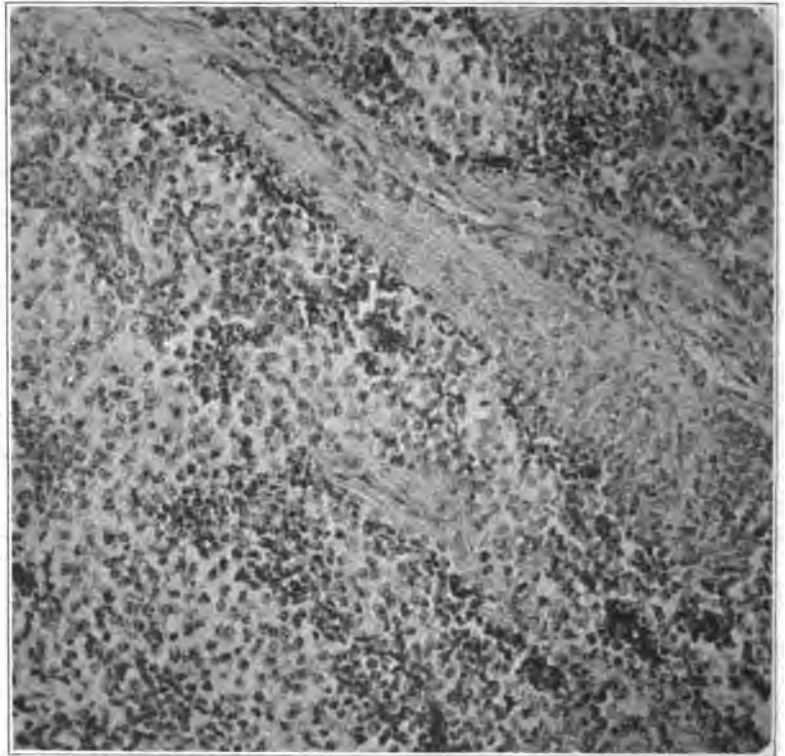
FIG. 3.   Diffuse endothelioma of bone. Compact structure of large poly-
hedral cells.

sheets of polyhedral cells without intervening stroma (Fig. 3). This origin, however, did not seem to be fully supported until I encountered sections in one case in which the cells were found to line a complex series of fine channels inclosing intact blood (Fig.
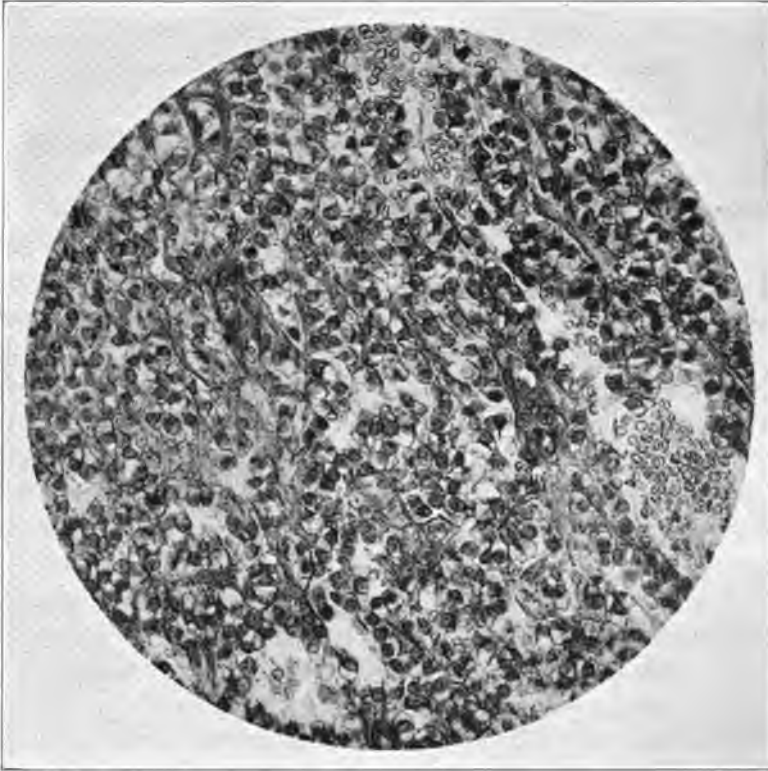
FIG. 4. Diffuse endothelioma of bone. Showing blood sinuses lined by tumor cells.

4). Here the endothelial character of the cells was quite pronounced, but they were much smaller than those occurring in angio-endothelioma, with which this tumor is doubtless closely related. In other portions of the same growth the cells appeared in diffuse sheets without capillary lumina, as seen in the other tumors of the series.

The exact point of origin of the growth is not clear, but the

early rarefaction of the bone indicates that the disease begins in the blood vessels of the bone tissue. Yet an involvement, simultaneous or early, of the vessels of the bone marrow can not be excluded. In the discussions of multiple endothelioma in the literature some authors thought they could trace the origin to the vessels, blood or lymph, of the periosteum. Many multiple endotheliomas, as in Marckwald's case, have appeared well within the bone marrow.

The designation of the tumor as endothelioma rather than as myeloma seems advisable, since myeloma is properly reserved for tumors derived from the specific cells of bone marrow.

The possible relation of the endothelial tumor to plasma cell or other forms of multiple myeloma deserves consideration, but the evidence at present available indicates that the two processes are distinct. I have found no definite plasma cells in any of the specimens. Plasma-cell myeloma is nearly always multiple and often very widespread. Bence-Jones protein has not appeared in any of the cases of endothelioma, but is often absent in myeloma. Multiple myeloma also perforates the bone rapidly and destroys it completely, while these tumors cause slow, rather diffuse rarefaction.

A relation to the angio-endotheliomas and other forms of endothelioma, solitary and multiple, described in the literature, must be assumed to exist. Most of these tumors accessible in the literature have occurred in adults and were clearly recognized as endothelioma. All the tumors of the present series have occurred in children, and with one exception they have been solitary.

The main point of the present communication lies in the demonstration that there is a rather common tumor occurring in young subjects, commonly identified with osteogenic sarcoma, and usually called round cell sarcoma, which is really of endothelial origin, and which is marked by such peculiar gross anatomical, clincal, and therapeutic features as to constitute a specific neoplastic disease of bone.

A CASE OF THYMOMA

JAMES EWING, M.D.

The case I have to present I call thymoma for want of a better term, rather than from any positive conclusion that it belongs in this category. The specimen comes from a man thirty-nine years of age, who began to suffer a little over three years before his death from swelling of the cervical lymph nodes, which was the only symptom that attracted attention. He had suffered from these swollen nodes for about a year before any attention was drawn to his chest. Then it was thought he had a mediastinal tumor. He was treated by various internal methods for another year, when he reached New York, and here the presence of a mediastinal tumor was demonstrated. He arrived at the Memorial Hospital about three months before his death. At that time he showed traces of the original enlargements of the cervical lymph nodes. These were reduced by radium and *x*-ray treatment, but the thymic tumor persisted. It was somewhat reduced in size, according to the *x*-ray plates, during his stay in the hospital, but did not disappear. At the beginning of his stay in the hospital abdominal palpation indicated that he had some swelling in the abdominal region, and these abdominal signs increased rapidly, so that after a time it was possible to demonstrate that the pelvis was filled with a tumor. This continued to grow, and all during this period he suffered from progressive anemia. He had no specific signs of myasthenia gravis. He died with symptoms of asphyxia and with a cachexia which went with the progressive anemia. A point that should be emphasized is that for at least two years he was regarded as a case of Hodgkin's disease.

At autopsy quite a remarkable condition was present. The

lymph nodes of the neck were not palpable. They were repre-
sented by small, pea-sized nodules, more or less sclerotic. In the
region of the thymus was a solid, rather soft, very opaque, light
yellow, but not distinctly lemon colored, tumor mass measuring
$6 \times 8 \times 5$ cm., capping the pericardium, composed of very numer-
ous, more or less discrete nodules, surrounding a considerable
portion of the trachea and the great vessels. The mucous mem-
brane over several inches of the trachea and bronchi showed sub-
mucous infiltration. The main mass appeared in the abdomen
and presented almost a continuous growth from the bottom of
the pelvis, surrounding the rectum, running up in a continuous
mass along the great vessels, adherent to the spinal column, sur-
rounding the kidneys, but limited by the renal capsule, surround-
ing the pancreas, which ran through the tumor without any signs
of compression, and running up into the nodes at the celiac axis.
It is difficult if not impossible to determine the origin of this
tumor. From the clinical standpoint the evidence is strongly in
favor of a primary mediastinal tumor, because during the ob-
servation of the patient before he reached New York, attention
had been specifically drawn to the presence of a mediastinal
tumor, and this was quite easily demonstrable by the x-ray plate.
It was only within the last three months of his life that the pelvic
and abdominal tumors could be recognized. A careful anatom-
ical study of the mediastinal growth gives only suggestive, but
not positive, evidence that we have a primary thymic tumor. The
next piece of evidence might come from the histological struc-
ture, but the tumor does not show the characteristic structure
presented by some thymic tumors. That structure shows lympho-
cytes in a peculiar stroma in which giant reticulum cells are very
prominent. The structure of this tumor presented a rather dif-
fuse growth of cells larger than lymphocytes, with large nuclei,
and with a strong tendency to fasten themselves to the strands of
connective tissue making up the stroma. I am convinced that
we are not dealing with a simple lymphosarcoma, either lympho-
cytoma or reticulum cell sarcoma, but I am not able to assert that
the tumor arises from the thymus. I think that the clinical evi-
dence and the anatomy of the disease favor the diagnosis of a

tumor of the thymus. The structure does differ from that of a simple lymphosarcoma.

One has to recognize three histological structures in thymic tumors. One has been called by the French observers "thymic carcinoma." It is made up of polyhedral cells, often alveolar in arrangement, and without any doubt derived from the epithelial reticulum cells of the gland. There is another tumor made up of lymphocytes derived from the lymphocytes of the thymus, and properly regarded as the thymic form of lymphocytoma. There is a third group, concerning the nature of which I am not prepared to make any dogmatic statements. It is a very cellular and malignant tumor composed of cells that are rounded, irregular, larger than lymphocytes, with large hyperchromatic nuclei showing a tendency to attach themselves to the stroma with a certain polarity. That structure does not belong to lymphosarcoma. I am inclined to think that we have here to deal with a tumor derived from the reticulum cells of the thymus, but more anaplastic than the ordinary carcinomas of the thymus, and hence growing more diffusely. If this conclusion is correct, then there are two types of tumors derived from the stroma cells, which are epithelial in origin, and one derived from the lymphocytes. These very cellular thymic tumors have been described in the literature, and they have regularly disseminated widely and produced bulky growths.

A REVIEW AND CLASSIFICATION OF BONE SARCOMAS

JAMES EWING, M.D.
NEW YORK

A REVIEW AND CLASSIFICATION OF BONE SARCOMAS

JAMES EWING, M.D.

NEW YORK

The knowledge of tumors of bone and bone marrow is still in the descriptive stage. To reach a correct histologic diagnosis of the case and to provide some conception of the probable clinical course are all that can be expected at the present time, and often more than is accomplished. The separation of the numerous tumors of this group into well-defined clinical and pathologic entities is far from complete, while knowledge of the exact origins of these tumors and of their various exciting and contributing causative factors is extremely fragmentary, or, indeed, wholly lacking. Moreover, with the present trend of medical research, the prospects are not favorable to important progress in this field. Serology, immunology, chemistry, studies in nutrition, and the use of modern instruments of precision have contributed little and promise little in the study of bone tumors. Abundant clinical material, wide clinical experience, and knowledge of the embryology, physiology and pathology of bone, are the more essential qualifications for successful investigation in this field.

Such studies call for clinical observation by the surgeon, and morphologic research by the pathologist, by each of whom the work must be carried out on a broad and systematic plan. The surgeon must provide · complete clinical pictures of the different neoplastic diseases of bone; and the pathologist must discover the origin, the predisposing anatomic conditions, the mode of growth, and the general etiologic factors, if an effective knowledge of these diseases is to be attained. When this knowledge has been secured, it will still be necessary to devise means for its dissemination so that it may become effective in controlling the mortality and possibly the incidence of these diseases.

For this purpose a uniform nomenclature is highly desirable or essential; and the main clinical and morphologic features of the different bone tumors must be clearly presented in accessible form. The present article is intended as a contribution toward this end.

CLASSIFICATION OF TUMORS OF BONE AND BONE MARROW

In the legitimate classification of tumors of bone only those neoplastic diseases which clinical experience shows actually to occur must be

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recognized. To divide the tissues into their constituent cells and to classify tumors blindly according to the possible cells of origin is unsound and unserviceable. Most tumors are not simple structures composed of a single cell, but are complex and become simple growths only in anaplastic metastases. Moreover, the tumors do not develop at random. They arise under quite definite conditions, which usually determine their course, and these conditions must be considered in a classification. The classification must be both clinical and anatomic. If a central chondrosarcoma arises from an aberrant superfluous island of cartilage misplaced by a rachitic process, then this disease may be recognized as a specific entity. If an osteogenic sarcoma arises at an epiphyseal line from traumatic partial separation of the epiphysis, or because of some structural defect, it also becomes a specific entity which deserves to appear in classification. On the other hand, if in the foregoing examples, considerable variations in structure of the tumors are observed in different cases which run much the same clinical course, it is unwarranted to subdivide such tumors because of minor structural variations, since they remain essentially the same diseases in origin, course, and general significance. The practice of designating bone tumors simply as round, spindle, or giant cell is quite inadequate to define the disease properly. Nearly all malignant tumors show all these types of cells at different periods or in different portions. When, however, the disease has been identified, for example, as osteogenic sarcoma, the further designation of cell type (spindle, etc.), is useful to indicate the degree of anaplasia or potential malignancy of the tumor. To a considerable extent, it is possible to classify bone tumors according to the foregoing plan, while increasing knowledge will serve to correct errors as they come to light. Only such a classification will be of service to both clinician and pathologist. Accordingly, one may recognize the forms of neoplastic diseases originating in bone and bone marrow given in the accompanying classification.

CLASSIFICATION OF BONE TUMORS

Osteoma { Spongy / Ivory

Chondroma { Pure chondroma / Chrondromyxoma / Myxoma } { Capsular / Periosteal / Central

Angioma : Cavernous

Endothelioma { Angio-endothelioma / Diffuse } { Solitary / Multiple

Benign Central Giant Cell Tumor and Its Variants { 1. Bone cyst / 2. Giant cell tumor / 3. Xanthosarcoma / 4. Myxosarcoma (benign)

Osteogenic Sarcoma
{ Periosteal (extraperiosteal)
Solid medullary and subperiosteal
Telangiectatic
Sclerosing

Myeloma
{ Plasma cell
Lymphocytic
Myelocytic
Erythroblastic

The inclusion of pure myxoma under the heading chondroma appears to be indicated because of the great probability that all pure myxomas of bone are derived originally from cartilage. The myxomas may be more difficult to eradicate surgically than chondromas but they do not exhibit any greater potential malignancy. Under the benign giant cell tumors, which must usually be regarded as sequelae of osteitis fibrosa cystica, are included for convenience sake, simple bone cysts. These lesions are not tumors; but Martland has shown that the simple cyst may result from the spontaneous degeneration and softening of a giant cell tumor.

The existence of telangiectatic osteogenic sarcoma as a distinct disease has not yet gained general acceptance. However, I am convinced that this lesion is one of the most specific of bone tumors, both in its gross anatomy and in its clinical course. One type of malignant bone aneurysm is a vascular osteogenic sarcoma. Its possible relation to cavernous angioma must be considered. In spite of recent contributions to the contrary I believe that the existence of four specific types of myeloma is firmly established. Wallgren,[1] however, concludes that all myelomas arise from a single primitive marrow cell. Of the groups of tumors mentioned above, I shall discuss only the cellular forms in the present paper.

Outline of the Clinical, Anatomic and Structural Features
of Some Common Bone Tumors

ENDOTHELIOMA

The knowledge of endothelioma of bone has reached a stage which calls for definite recognition of this disease. The condition is generally submerged under the general diagnosis of round cell sarcoma or myeloma, since both are composed of small diffusely growing cells with round nuclei. Having seen ten cases within the past year, I cannot regard it as a rare disease. Three anatomic forms may be recognized: (1) multiple endothelioma; (2) solitary angio-endothelioma, and (3) diffuse endothelioma.

1. Multiple endothelioma, in which nearly every bone in the body was the seat of small medullary tumors, occurred in a remarkable

1. Wallgren: Virchows Arch. f. path. Anat. 232:381, 1921.

case described by Marckwald. It is the only recorded case of its
kind. Symmers and Vance [2] have described a somewhat similar case, and
multiple tumors in much smaller numbers have often been observed.
In several of the cases collected by Howard and Crile, there were

Fig. 1.—Angio-endothelioma of bone.

multiple tumors. Whether the several tumors were primary or secon-
dary and metastatic from one original growth cannot be determined;
but multiple primary tumors undoubtedly occur. They appear in adults

2. Symmers, D., and Vance, M.: Am. J. M. Sc. 152:28 (July) 1916.

and nearly all have been fatal. Metastases in lungs and lymph nodes occur. It does not appear that any have been treated by modern roentgen-ray technic. The diagnosis rests on the roentgenogram, which shows multiple central tumors with diffuse absorption of bone. Multiple myelomas are much more numerous and sharply perforate the bone. The structure consists of endothelial-like cells in small groups or sheets, often forming alveoli and sometimes cysts containing serous or mucinous fluid.

Wells has recently described a multiple endothelioma in which isolated small groups of cells, similar to those described by Marckwald, appeared, which seemed to be derived from the blood vessels of atrophic and mucinous bone marrow.
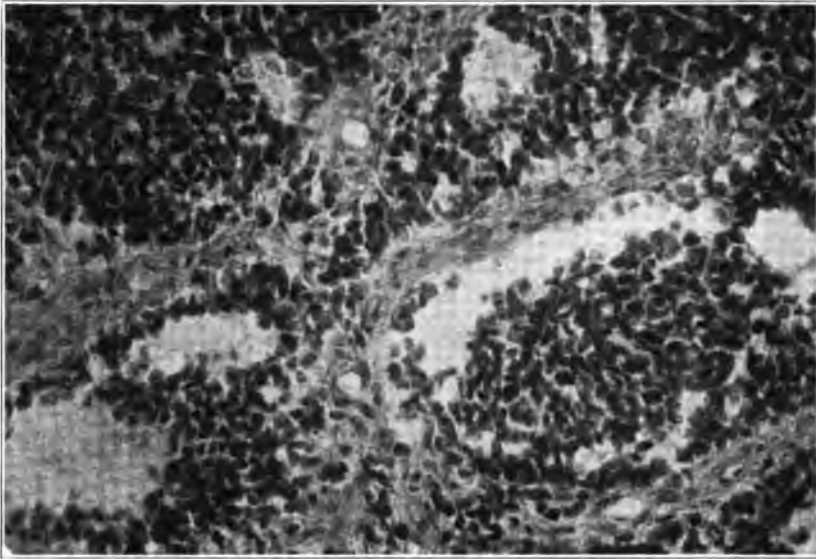
Fig. 2.—Structure of angio-endothelioma of bone.

2. Angio-endothelioma usually appears as a single tumor which sharply and completely destroys the bone and often reaches large dimensions (Fig. 1). Its expansile pulsation places it among the malignant bone aneurysms. The roentgen ray shows a clean cut destruction of bone by a central tumor which displaces and eventually invades the soft parts. There is no bony capsule and the periosteum is free. This disease returns after amputation and produces metastases. It grows much more rapidly than the benign central giant cell tumor, but can hardly be distinguished from telangiectatic sarcoma on clinical and roentgen-ray evidence. From the ordinary osteogenic sarcoma it differs in that it fails to show a wide zone of involved periosteum extending beyond the main tumor mass. These differences are rendered obvious by a comparison of the photographs of gross specimens with roentgen-

ograms. The structure is highly characteristic, consisting of large clear endothelial cells in cords and columns inclosing circulating blood (Fig. 2). The subjects are chiefly adults.

3. Solitary diffuse endothelioma occurs in young subjects, all of my patients being under 21 years of age, five of them being 14 years old. The bones involved were: ulna, twice; radius, once; tibia, twice; scapula, once; skull, once; pubes, twice, and femur, once. It begins slowly, with pain and gradually developing disability. After several months, spontaneous fracture may occur. Transient variations in size of the tumor have been noted and may prove very deceptive both to the patient and to the physician. One patient died from metastases after amputation. Another developed multiple tumors of the skull. Bence-Jones protein was found in one case only. The tumors develop in the marrow or in the bone or in both. The shaft is widened and slowly absorbed without a trace of bone production. The soft tissues are gradually invaded. The most striking feature, best revealed by the roentgen ray, is the involvement of a large segment or the whole of the shaft, which distinguishes the process from osteogenic sarcoma and the giant cell tumor. This location, together with the smooth fading of a slightly widened shaft, generally permits a diagnosis from the roentgeno-gram alone (Fig. 3). The differential diagnosis from the benign giant cell tumor and from osteogenic sarcoma is assured if the tumor recedes rapidly under roentgen-ray or radium treatment. True myelomas, how-ever, may respond in the same manner. The structure presents diffuse sheets of small polyhedral cells with clear cytoplasm, without inter-vening stroma, often undergoing mucoid or hydropic degeneration (Fig. 4). Zenker's solution best preserves the cell form. After poor fixation, the tissue resembles that of a myeloma or "round cell sarcoma." That the cells are endothelial is shown by the appearance in some cases of many spaces lined by tumor cells and filled with intact blood. The structure may approach that of angio-endothelioma or it may verge on myeloma. Plasma cells are absent or scanty and uncertain; but the possible relation of this tumor to plasma cell myeloma remains to be determined.

The importance of recognizing this tumor lies in the fact that amputation is probably unnecessary, because the tissue is remarkably susceptible to the roentgen ray and radium. The prognosis is, how-ever, complicated by the occurrence of multiple tumors and metastases. One of my patients died with multiple tumors of the skull which were believed to be primary, and another is said to have died with pulmonary metastases after amputation of the arm.

The comparative frequency of this disease renders it one of the most important of bone tumors. Since the structure is quite different

Fig. 3.—Diffuse endothelioma of ulna.

from that of the easily recognized endotheliomas with blood channels, alveoli and cystic spaces, it appears clear that the cases are not recognized as endothelioma, but are classed as round cell sarcoma or myeloma. Since solitary myeloma is not a frequent diagnosis, most of the cases probably pass as round cell sarcoma. Unless the pathologist is familiar with the structural details of diffuse endothelioma, he will invariably render a diagnosis of round, or possibly small, spindle cell sarcoma, or myeloma, in these cases. I have been making this error myself for many years, although I was long aware that the tumors did not correspond to any known form of bone cell or bone marrow cell neoplasm. That the tumors are not osteogenic sarcoma became apparent when · their prompt recession under radium was
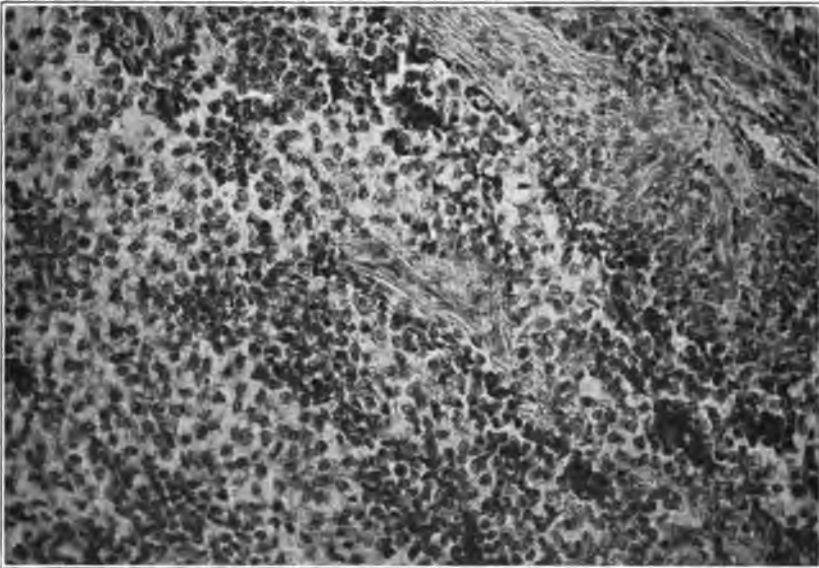
Fig. 4.—Diffuse endothelioma of bone; showing compact structure of large polyhedral cells.

observed, for oseogenic sarcoma reacts slowly to radium. That the growths are of endothelial nature was strongly suggested by the appearance of broad sheets of clear polyhedral cells without a trace of intercellular stroma. This origin was finally demonstrated by the observation of a case in which the usual diffuse structure was present in much of the material removed at exploratory operation, while in other portions the growth was composed exclusively of blood spaces filled with intact blood and lined by large clear endothelial cells.

The apparent limitation of the disease to young patients is one of its most interesting and suggestive features. Of the twenty cases of

endothelioma collected by Howard and Crile,[3] three occurred in patients under 20 years of age. One of these in the femur of a girl of 9 years, with other tumors in the skull, approached in structure the diffuse type, the blood spaces being lined by from five to ten rows of cells. In Kolaczek's patient, 18 years of age, a very large tumor of the tibia was in part composed of spindle cells, and in part of alveolar endothelioma. Crile's patient, at 11 years, showed alveoli and well defined lymph spaces. As Crile points out, the angio-endotheliomas occur with few exceptions after the period (40 years) when osteogenic sarcoma is most frequent. They often develop in patients of quite advanced years, 50 to 75, when osteogenic sarcoma is practically unknown.

These observations regarding age incidence throw no light on the nature of the underlying conditions in the bone and marrow which lead to endothelioma; but they show that endothelioma yields somewhat different results at different periods of life.

Much further exploration of the dark territory of the general pathology of bone and bone marrow is needed before one can formulate any hypothesis regarding the conditions of origin of these tumors. Most of the young subjects show a secondary anemia, without specific characters. Usually the skeleton is delicately built. A history of rickets or scurvy or other notable disorders of childhood has not been elicited. Six of my ten patients were Jews. One was very fat, as were also his two brothers. Signs of status lymphaticus were not definitely present.

Regarding the choice of treatment of this disease, I wish to emphasize the caution, that, while the diffuse endotheliomas of young subjects have proved uniformly susceptible to heavy radium packs and to repeated applications of roentgen ray, sufficient time has not elapsed to determine the final outcome of this treatment. The tumors tend to recur unless treatment is continued over a long period. The danger of metastases during treatment must be considered. On the other hand, the tendency to produce metastases appears to be less prominent than in the case of osteogenic sarcoma. Many of the multiple tumors are probably primary, so that amputation is often futile. Hence it seems justifiable or even strongly indicated to immobilize the member and attempt to save the limb or life by physical therapy. If this plan is adopted, it is highly important to avoid incision into the tumor. The diagnosis can generally be made on the clinical history, the peculiar roentgenogram, and the early response to radium. It may be of interest to note that competent observers firmly maintained that some of my cases were osteomyelitis, while one patient was persistently treated with arsphenamin.

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3. Howard and Crile: Ann. Surg. **42**:358, 1905.

BENIGN GIANT CELL TUMOR AND ITS VARIANTS

In 1852, Nelaton and Robin, in a well illustrated monograph, clearly pointed out the gross, microscopic and clinical features of this benign *tumeur à myeloplacques,* contrasted it with the malignant osteogenic sarcoma, and urged conservative treatment. Gross, also, in 1868, clearly described the disease and recognized its benign nature. These contributions seem not to have been widely read; but several later writers, especially in America, have described the disease and discussed it as an important novelty. It is quite apparent also that many surgeons and pathologists are still unfamiliar with the important differences that separate this disease from osteogenic sarcoma, so that many limbs have been unnecessarily sacrificed and many supposed bone sarcomas have been erroneously assumed to be operative cures.

The knowledge of the clinical and morphologic characters of the common benign central giant cell tumor we owe chiefly to Nelaton and Robin; but it has long been felt that the scope of these tumors is inadequately defined. Some of them do not behave like benign neoplasms. Gross believed that he observed violations of the rule that they never produce metastases. Many of them recur after operation and infiltrate the soft tissue or even break into the joints. Also the microscopic diagnosis occasionally presents serious difficulties, even for the experienced observer. It is the variants of the disease which give rise to difficulty.

The typical forms of the disease seem to find their origin mainly in the changes produced by osteitis fibrosa cystica; and the simple bone cyst is usually found as a part of localized osteitis fibrosa. Osteitis fibrosa affects one or many bones and produces a change in the marrow marked by diffuse overgrowth of spindle cells, loss of hematoblastic and fat cells, absorption of cancellous tissue and often thinning of the shafts. About the dissolving bone trabeculae and along the inner surface of the shaft, numerous giant cells are often found. Cysts form in the new tissue, and these may be lined by giant cells and filled with serous fluid. Hemorrhages readily occur in the cysts and the inflammatory reaction about these blood masses leads to the growth of granulation tissue in which are often many giant cells and disintegrating bone. In some such manner the ordinary giant cell tumor probably develops; but it must be admitted that the exact mode of origin of the simple tumors has never been certainly traced. Martland [4] has shown that simple cysts may result from the liquefaction of previous giant cell tumors. I have evidence that some giant cell tumors result from the absorption of aberrant islands of cartilage with neoplastic proliferation of the released cartilage cells, and others. It appears

4. Martland, H. S.: Proc. New York Path. Soc. **21**:102, 1921.
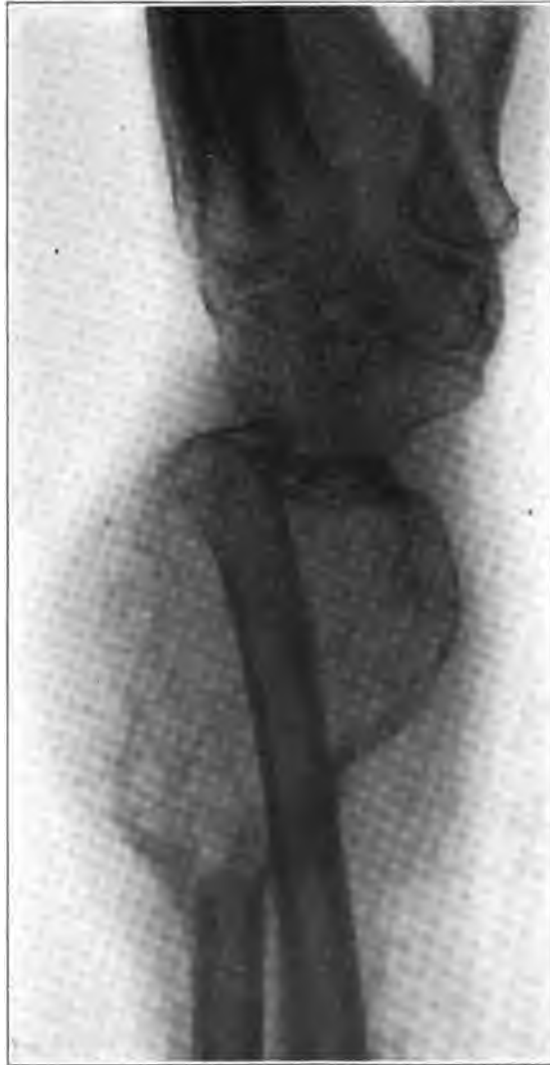
Fig. 5.—Benign central giant cell sarcoma, epulis type; showing limiting shell of bone and multicystic appearance.

also that any simple hematoma of bone marrow may lead to the production of granulation tissue with many giant cells. Barrie's designation "hemorrhagic osteomyelitis" would not be inept for some of these processes if it did not suggest an intense inflammatory reaction. This array of possible initiatives of the peculiar tumor tissue is not inconsistent with the variety of clinical conditions under which the disease is observed. The benign giant cell tumor is of wide occurrence as respects age, location, multiplicity, and previous history of the patient, and it is unlikely that it always arises under identical conditions.

1. *Typical Benign Central Giant Cell Tumor.*—This is a characteristic condition. It occurs usually as a single tumor in one end of the tibia, fibula, humerus, femur, lower jaw, or lower end of the radius, or in the flat bones. It progresses slowly, with pain, swelling and disability. Spontaneous fracture may result. The soft parts are displaced. It usually begins near the epiphyseal line, where the cancellous tissue is absorbed and the shaft widened by a globular mass. A thin, sometimes crackling, shell of bone is regularly laid down about the advancing tumor.

The roentgenogram shows a multicystic appearance, with destruction of the shaft, an irregular deposit of outlying shell of bone and sharp limitation from the soft tissues. Occurring in the middle of a long bone, there may be a sharp irregular fracture with comparatively little widening. Beyond the tumor the periosteum is unaffected (Figs. 5 and 6).

On gross examination such tumors are nearly always found to be cystic; or the central portion is diffluent and filled with blood detritus, chocolate colored fluid, or clear serum. The texture of the growth is that of soft, reddish, vascular granulation tissue, becoming more and more dense toward the periphery.

The structure shows an abundance of giant cells with many small separate nuclei. They appear in masses or they surround capillaries or blood spaces. They are derived from the vascular endothelium but participate in the tumor process, sometimes extensively. The stroma is composed of many capillaries supported by a moderate number of spindle fibroblasts with nuclei showing normal or slightly increased chromatin (Fig. 7). Tumors of this type are always strictly benign, in the oncologic sense, although they may lead to serious clinical disturbances. They may be cured by curettage, by roentgen ray and radium, and some of them disappear spontaneously. They may become transformed into simple cysts. They are prone to become infected from curettage or exploratory incision, and a progressive cellulitis and osteomyelitis may develop. The wide cavities left after curettage may offer some surgical problems.

Fig. 6.—Benign giant cell tumor of upper end of tibia; collapse of joint surface without invasion of joint cavity; infection through line of diagnostic incision.

2. *Xanthosarcoma.*—Some of the central giant cell tumors of bone
are solid throughout, firm, dry, and yellow. The yellow color is due
to the presence of many large polyhedral cells distended with lipoid
granules. This is a structure commonly designated as xanthoma (Fig. 8).
These tumors have greater growth capacity than the ordinary giant
cell tumor. They often reach considerable size, breaking down the
bony capsule and forcing their way between muscle and fasciae,
but definite infiltrative growth is rare. They may destroy the epiphysis
and lead to collapse of the joint surface. The roentgenogram reveals
an incomplete bony capsule, or none.
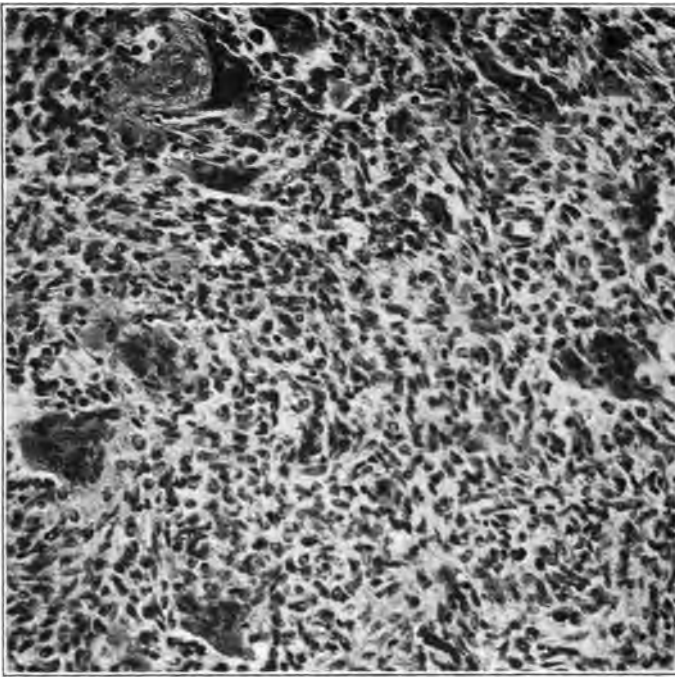
Fig. 7.— Structure of benign giant cell tumor; polynuclear giant cells
lying among spindle and polyhedral cells; slight hyperchromatism of nuclei.

The structure presents a diffuse growth of medium sized spindle
cells with very little hyperchromatism. Varying proportions of the
tissue are composed of the lipoid cells. Giant cells are missing over
large portions of the growth; but usually they appear abundantly
in some portions, generally about blood spaces or blood extravasations.
If the histologist encounters only the spindle cells, an erroneous
impression of a malignant tumor is liable to be gained.

These tumors are prone to recur after curettage, especially when
large, owing to the difficulty of reaching all portions of the growth.

Infection is also a frequent complication which results in the loss of many limbs and of some lives. They slowly respond to roentgen-ray and radium treatment; but one who chooses this method of treatment must plan to restrict the motion of the part and devote some months to the treatment. Immobilization reduces blood supply, avoids the danger of fracture, and permits weakened joint surfaces to remain intact.

I have never known these tumors to produce metastases, and I have been unable to find authentic records of such complication; but it seems quite possible that by curettage groups of viable cells could be dislodged from the tumor and pass into the blood vessels. However,
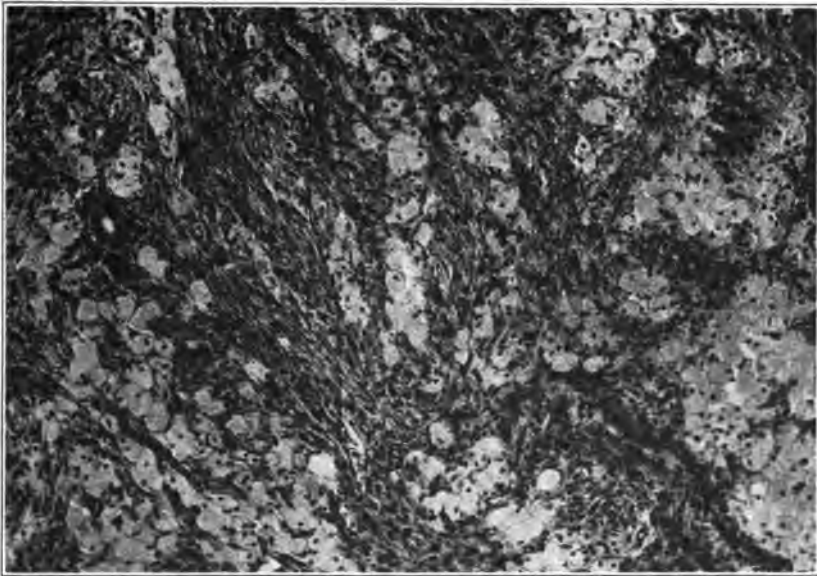
Fig. 8.—Benign giant cell tumor; peripheral portions showing xanthosarcoma; other portions showed many typical giant cells of epulis type.

the blood vessels are usually small and scanty, and the danger of metastases can probably be ignored.

That the central xanthosarcoma of bone is a variant of the giant cell tumor is clearly indicated by the clinical history, central location, general structure, the course of the growth as revealed by the roentgen ray, and by the not infrequent occurrence of xanthoma cells in typical giant cell tumors. In the benign tumors of tendon sheaths xanthoma cells are generally prominent.

3. *Myxosarcoma.*—The peripheral portions of many giant cell tumors are often composed of solid elastic opaque tumor tissue of myxoma-

like structure, while the central area alone is soft and vascular and contains the characteristic giant cells. Even this central area may be reduced to a minimum, and nearly the whole tumor may be composed of solid opaque tumor tissue in which there are many spindle cells lying in a mucinous matrix (Fig. 9). Although the central location and rather sharp limitation of the growth strongly indicate a benign character, the histologic structure with many spindle cells suggests malignancy, and this impression may be strengthened by reliance on frozen sections. However, these central tumors are benign and their
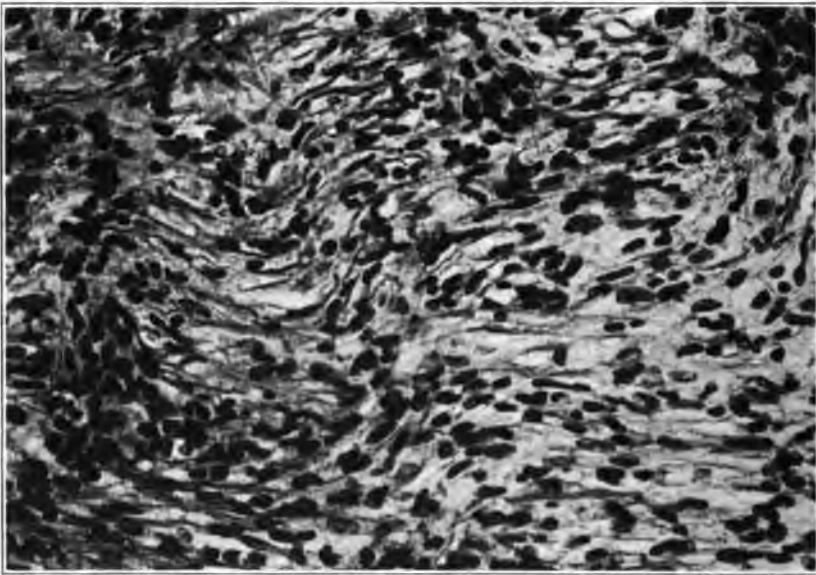
Fig. 9.—Benign giant cell tumor; peripheral portions composed of large spindle cells with clear cytoplasm; other portions showed giant cells of epulis type.

virtual identity with the ordinary giant cell tumor is shown by the gross transitional forms which connect the two types and by the presence of typical giant cells which may be seen in some parts of the tumor. They are quite different in structure from the fascial myxosarcomas and from any myxomatous areas of osteogenic sarcoma.

In some cases the main tumor cell is large and spindle or polyhedral in form with vesicular nucleus, while giant cells are scanty. These spindle cells lack the hyperchromatism of the malignant osteogenic tumors. Numerous small lymphocytes may also appear diffusely or in clusters, which are probably derived from the lymphoid marrow and which are almost never seen in malignant sarcomas (Fig. 10).

4. *Giant Cell Tumors of Cartilaginous Origin.*—Occasionally, one encounters giant cell tumors containing islands, streaks, or larger masses of hyaline cartilage. Since there are no indications that the giant cell tumor can produce cartilage, one is forced to conclude that the tumor is associated with the absorption of misplaced islands of cartilage. Such misplaced masses of cartilage lying in the marrow cavity in the vicinity of the epiphyseal line are very frequently seen in rickets. All the tumors of this type that I have seen were found at the ends of long bones.
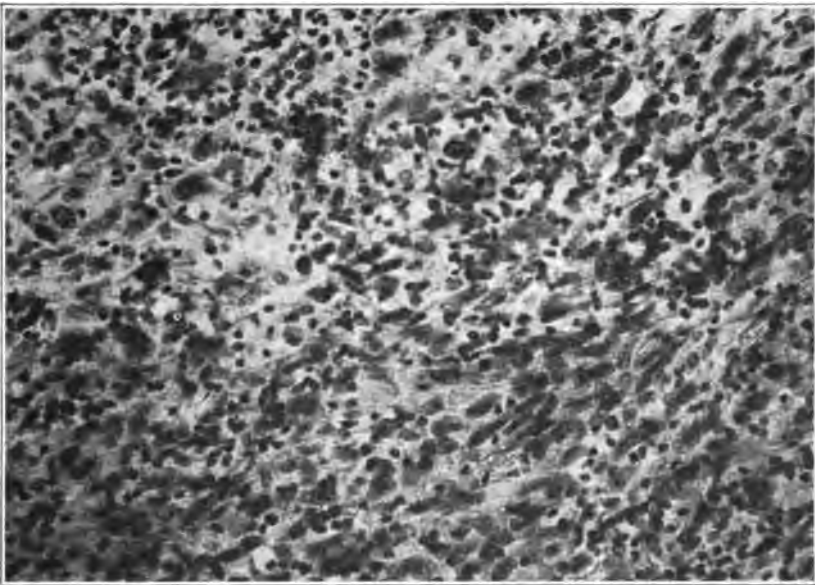
Fig. 10.—Benign giant cell tumor, edematous and infiltrated with lympocytes; tissue composed of polyhedral cells with clear cytoplasm; giant cells of epulis type in other portions of tumor.

They are of rather solid texture, opaque, sometimes slightly mucinous, and lack the reddish jelly-like features or the yellow color of the other forms. The giant cells are generally numerous and of the usual character. They lie among many smaller tumor cells which are often round or polyhedral, while the vascular spindle cell tissue of the ordinary giant cell tumor is missing. These round cells may grow diffusely over considerable areas in which giant cells are scanty or missing, so that the diagnosis of myeloma may be returned if only a small portion of the tissue is available for examination. Hence, as with many other bone tumors, a biopsy is a somewhat hazardous resort (Fig. 11).

. It seems probable that these growths represent a vigorous proliferation of tissue cells about degenerating cartilage. Probably the cartilage cells participate in this process as well as the endothelial and possibly other marrow cells. That the absorption of cartilage may be connected with other giant cell tumors in which no traces of cartilage or their peculiar derivatives are present, deserves, perhaps, further consideration. From their cellular character, one gains the impression that these tumors must be more malignant than the others; but several patients whom I have followed recovered after curettage. Being cellular they should be susceptible to radium and roentgen ray.
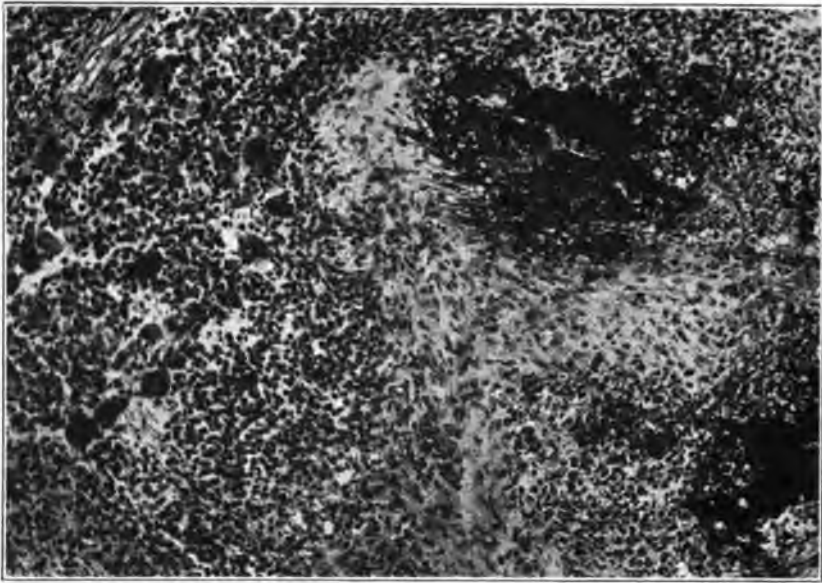
Fig. 11.—Benign central giant cell tumor with island of dissolving and partly calcified cartilage.

5. *Telangicctatic Giant Cell Tumors.*—In 1894 a portion of a central giant cell tumor was submitted to Dr. T. M. Prudden at Columbia for diagnosis and prognosis. It came from a growth which occupied a large lower segment of the shaft of the tibia in a girl, aged 14 years. The tumor was described as extremely vascular. After much discussion, on account of the presence of numerous typical giant cells, the tumor was pronounced benign. Curettage was recommended and performed. Several years later we were informed that the patient recovered, and that her leg was sound.

The structure showed very wide blood spaces separated by thin strands of spindle cell tissue lined by many giant cells. The spindle cells resembled those of the ordinary giant cell tumor.

That the course of central telangiectatic tumors with giant cells is not always favorable is shown by a case illustrated by Figures 12 and 13. The subject was a child of 10 years, in whom the head of the humerus was completely excavated by a bloody mass in which no tumor tissue could be detected on gross examination. Section revealed thin strands of tumor tissue composed of spindle cells with hyperchromatic nuclei. These inclosed wide blood spaces lined by numerous giant cells with multiple small nuclei which again were relatively hyperchromatic. Interscapular-thoracic disarticulation was performed; but the patient

Fig. 12.—Telangiectatic osteogenic sarcoma of head of humerus; malignant bone aneurysm, in a child of 10 years; pulmonary metastases.

is said to have died of pulmonary metastases four months later. This was not confirmed by necropsy. This case may well be classed as a malignant bone aneurysm. On account of this observation it seems proper to urge that more attention should be paid to the main tumor tissue than to the giant cells in the diagnosis of doubtful cases of this type.

6. *Borderline Cases of Giant Cell Tumors.*—Some bone tumors, mainly central in location, are found to have involved much of the neighboring bone shaft which is eroded or perforated, while the tumor tissue early penetrates the soft tissue and fails to throw out a uniform

limiting shell of bone. This anatomic condition is not easily distinguished in the roentgenogram from some osteogenic sarcomas; but careful palpation, roentgenograms taken from several angles, and surgical exploration reveal an absence of the extensive involvement of periosteum which is nearly constant in osteogenic sarcoma. The periosteum in these cases is generally quite intact beyond the main tumor mass.

The structure consists of numerous rather large spindle cells with vesicular nuclei which exhibit some, but not pronounced, hyperchromatism. Intercellular strands are scanty or absent. By hydropic imbibition the cells may appear polyhedral. Giant cells are scanty or absent in many areas but appear in groups, often in the clefts or
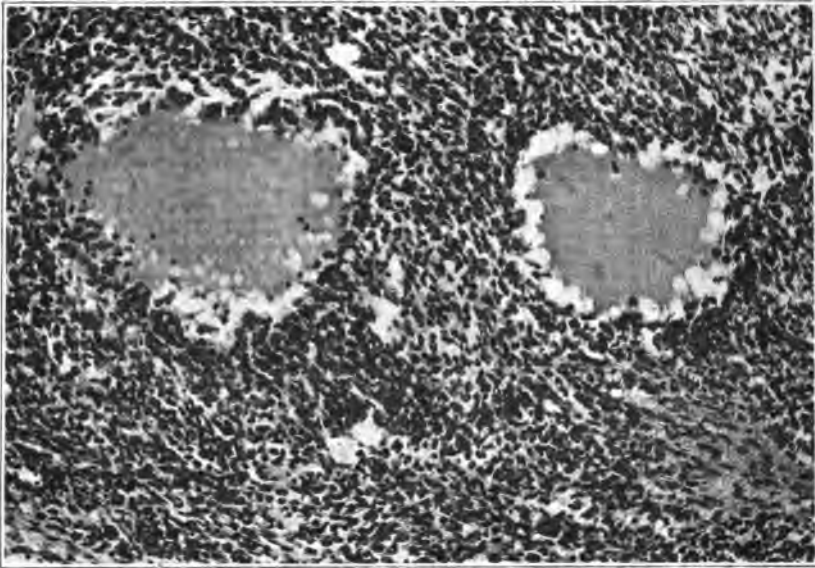
Fig. 13.—Structure of a telangiectatic osteogenic sarcoma; tissue from the outer shell of a highly vascular tumor; round and spindle cells with hyperchromatic nuclei enclosing blood sinuses; same case as Figure 12.

sinuses of tumor tissue. They are not so large as the giant cells of the ordinary giant cell tumor, and the nuclei, while multiple, are larger and more hyperchromatic. The diagnostic difficulties are increased when only curetted fragments of the tumor are available. Such tumors offer a difficult problem for the pathologist and for the surgeon. My colleagues and I have adopted the policy of designating these tumors as borderline tumors of the giant cell type and of giving a guarded prognosis. They are prone to recur after curettage; but I have not known them to produce metastases even after repeated insults.

While they probably belong with the benign giant cell tumor and are connected with the absorption of bone or cartilage, it seems possible that osteoblasts may participate in the proliferation and endow the process with more aggressive qualities than belong to the strictly benign tumors (Fig. 14).

7. Secondary Giant Cell Structure in Malignant Osteogenic Sarcoma.—Since the peculiar giant cells of the benign tumor occur whenever bone is being absorbed and since these structures signify in general a type of foreign body giant cell, one should be prepared to find them in malignant osteogenic tumors surrounding blood extravasations, or following infection, or surgical trauma. Under such
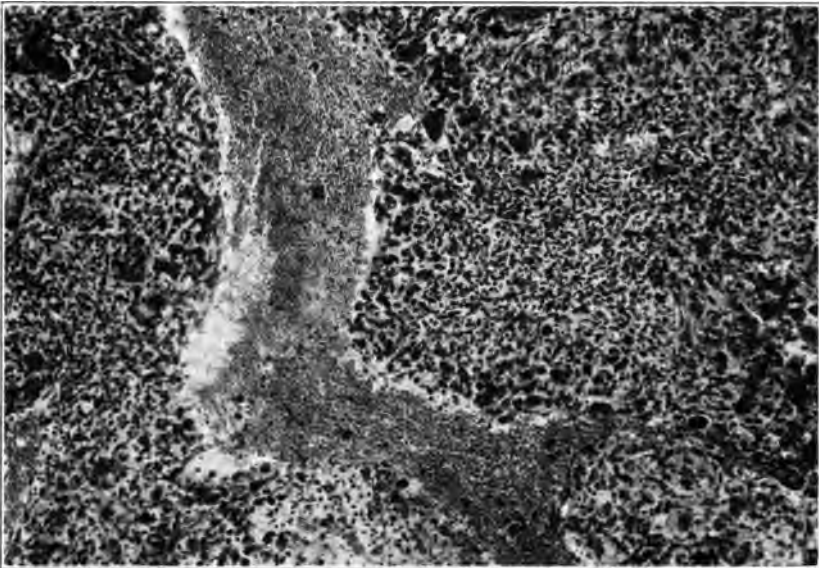
Fig. 14.—Benign giant cell tumor, low magnification; giant cell with many compact nuclei; polyhedral and spindle cells; many small lymphocytes.

conditions they not infrequently appear in malignant tumors and may lead to an erroneous diagnosis by the pathologist when only small portions of tissue are available. These and other considerations have led me to take the position that cutting into bone tumors and removing a small piece for diagnosis is generally hazardous. The diagnosis of bone tumors can safely be attempted only when all of the clinical history and roentgenograms are available. With such data carefully analyzed, the probatory incision is usually found to be unnecessary; and it is not too much to say that the gross anatomy of the lesion is often a safer guide to a correct clinical conception of the disease than the variable and uncertain structure of a small piece of tissue. Doubt-

less there are cases in which both classes of evidence are needed; but they constitute a minority of the group and the microscopic evidence is often indecisive.

The foregoing review of the conditions under which giant cells of the epulis type may be found in bone tumors reveals the difficulties which surround microscopic diagnosis in this field. When the giant cells form the major portion of the tumor, the process is almost invariably lacking in aggressive growth and clinical malignancy; but when they become scanty and are associated with many spindle cells and compact polyhedral cells, the tumors as a rule are more aggressive, while in some cases in which the giant cells are associated with other, more numerous, tumor cells of malignant histologic character the tumors, as in the malignant bone aneurysm cited, are quite malignant. In addition, there is the occasional occurrence of epulis giant cells in inflamed and degenerating osteogenic sarcoma. Under these circumstances, it seems desirable to restrict the importance of the presence of giant cells in the diagnosis of bone tumors, and to emphasize more strongly the importance of the main tumor cells. Furthermore, it seems probable that further study of the origin of these benign central tumors will lead to the definite subdivision of the group based on exact histogenesis and general etiology. The demonstration of a group of these tumors as probably derived from the absorption of cartilage is perhaps a step in this direction. The xanthomatous tumors also are peculiar in many respects and suggest that they too enjoy some peculiar conditions of origin. Osteitis fibrosa seems to be associated only with the benign vascular cystic tumors composed chiefly of giant cells of large size.

A more satisfactory term should be found to designate this group of tumors, and one which lays less emphasis on the giant cells. Central osteogenic sarcoma or benign central sarcoma may be suggested as worthy of consideration.

OSTEOGENIC SARCOMA

Of this, the main malignant tumor of bone, four distinct groups should be recognized, because of their peculiar gross anatomy, microscopic structure and clinical course. These are: (1) periosteal sarcoma; (2) subperiosteal and medullary sarcoma; (3) telangiectatic sarcoma, and (4) sclerosing osteogenic sarcoma.

I have previously urged the recognition of only three groups of osteogenic sarcoma, merging the first two groups of the present list for the sake of simplicity; but the facts demand the sacrifice of simplicity in the interest of more accurate knowledge. A large number of bone tumors are *extra*periosteal, while many others are *sub*periosteal and invade or originate in the marrow cavity. On dissection these tumors are found to be quite different in gross anatomy; in structure,

Fig. 15.—Malignant ossifying extraperiosteal sarcoma, which produced pulmonary metastases.

they are quite divergent, and the clinical courses are far from parallel. These contrasts suggest essential differences in the conditions of origin of the tumors.

1. *Periosteal Sarcoma.*—The extracortical position of these tumors is quite characteristic. In fact, the true periosteal sarcoma appears to originate from the periosteum itself or from the outer layers, leaving

Fig. 16.—Extraperiosteal osteogenic sarcoma; same case as that shown in Figure 15.

the bone shaft intact or slightly eroded, whereas other osteogenic sarcomas are subperiosteal and grow beneath the periosteum, separating it from the shaft over a wide area by a fusiform mass of cellular tumor tissue. These relations are indicated in the accompanying sketch (Fig. 20), and they become quite apparent in Figures 15, 16, 17 and 19.

Fig. 17.—Malignant spindle cell extraperiosteal and capsular sarcoma, fungating through the skin at line of diagnostic incision; infection with rapid enlargement from inflammatory edema.

The roentgenogram shows an intact or slightly eroded shaft running through a solid tumor mass which lies at one side and which is attached to a segment, often narrow, of the periosteum. Some of these tumors arise mainly from the capsule of the joint, but a pure capsular sarcoma is rare.

The small area of periosteum involved and the firm encapsulation frequently observed suggest that in such cases an effort may be made at local excision. Some periosteal sarcomas are dense fibrosarcomas which recur locally but which have only a moderate tendency to produce metastases. They also suggest conservative treatment.
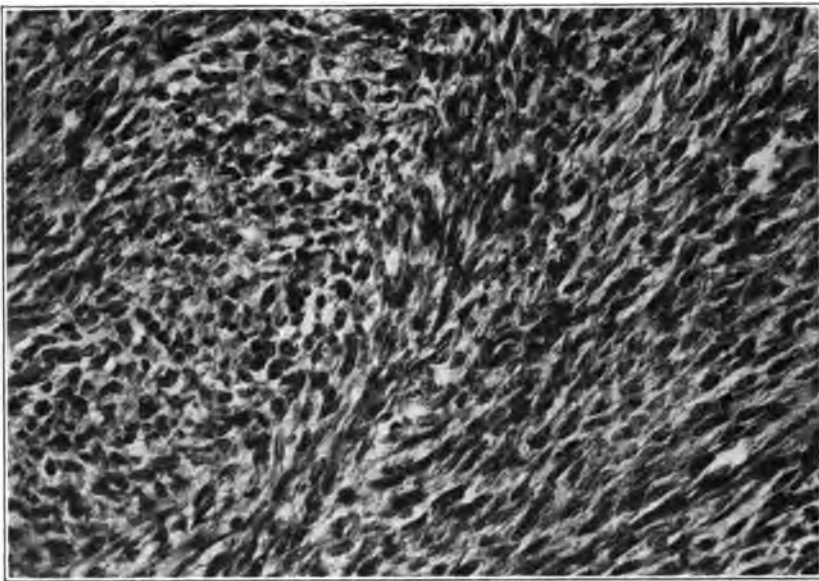
Fig. 18.—Structure of extraperiosteal and capsular osteogenic sarcoma; same case as Figure 17.

Most periosteal sarcomas produce bulky rounded solid growths which long remain encapsulated, pushing the soft parts before them. When forming cartilage, they may reach enormous dimensions.

The texture of periosteal sarcomas varies. Some are cellular, soft, crumbly, rapidly growing and extremely malignant. A tumor of the humerus gave myriads of metastases in nearly every portion of the body from calvarium to heel, while the pancreas was completely replaced by tumor tissue, composed of small spindle cells. A cellular tumor of the patella proved fatal six weeks after the originating injury was received.

Most of the tumors are firm because of the presence of much intercellular stroma, which appears in the form of stalactite strands of

hyaline, osteoid or osseous tissue, or masses of cartilage. The metastases may produce bone.

The structure of the periosteal sarcoma is usually specific and easily recognized. It presents spindle cells, small or of moderate size,

Fig. 19.—Solid central and subperiosteal osteogenic sarcoma; the sharp limitation at epiphyseal line may be noted.

but usually presenting hyperchromatic nuclei. Cell bodies are not always easily demonstrable, so that some of these tumors may pass as round cell sarcoma. In other cases the spindle cells are quite large,

and with increasing stroma they tend to become large and sometimes polyhedral.

Differing markedly from these malignant cellular growths is a series of cellular fibrosarcomas in which hyaline and fibrous stroma exceeds the bulk of the cells. Such tumors are not, as a rule, malignant. They recur locally after excision, but are slow to produce metastases. They probably form the majority of cures of osteogenic sarcoma by amputation (Figs. 21 and 22).
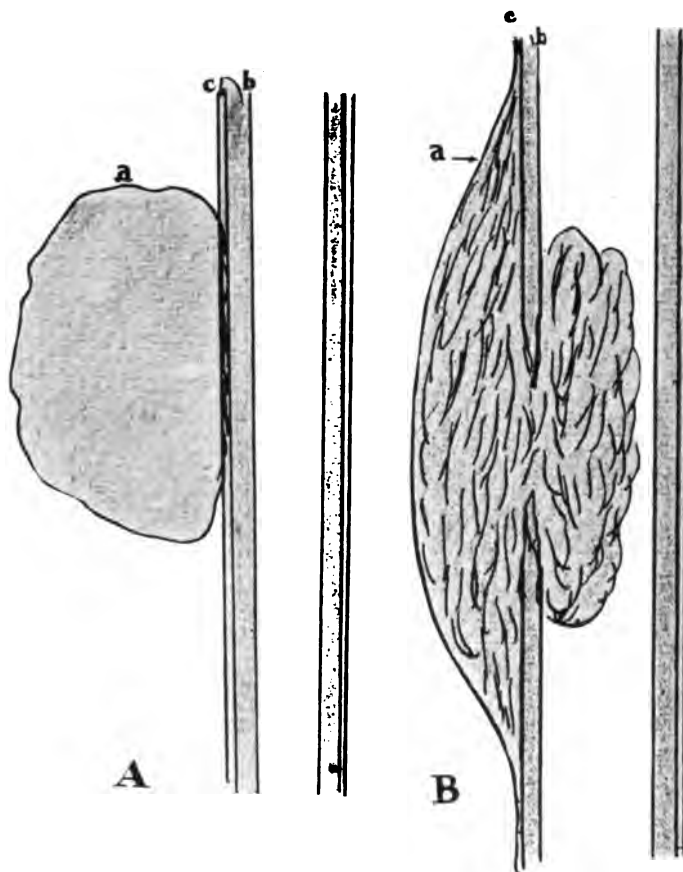
Fig. 20.—Diagram contrasting the relations of the extraperiosteal sarcoma and medullary and subperiosteal sarcoma: *A*, periosteal sarcoma; *B*, medullary and subperiosteal sarcoma; *a*, tumor; *b*, shaft and *c*, periosteum.

The diagnosis between hyperplastic callus and productive periostitis is not always easy, and must be based on general familiarity with both processes. Mistaking exuberant callus and productive periostitis for sarcoma is a very common error which may be avoided if the microscopist will insist on having unmistakable neoplastic characters

before concluding that a malignant process exists. Malignant bone-producing tumors very rarely develop within three weeks after a trauma. Periostitis usually covers a wide area, while tumors are localized.

2. *Solid Subperiosteal and Medullary Sarcoma.*—The most frequent form of osteogenic sarcoma involves marrow, shaft and subperiosteal tissue. The cancellous tissue toward the end of the diaphysis is converted into a solid tumor mass which extends up the marrow cavity, and after considerable delay may cross the epiphyseal line into the epiphysis. At the same time the shaft is destroyed and the tumor spreads beneath the periosteum, producing a fusiform swelling which gradually encases the bone. In the earliest cases which I have
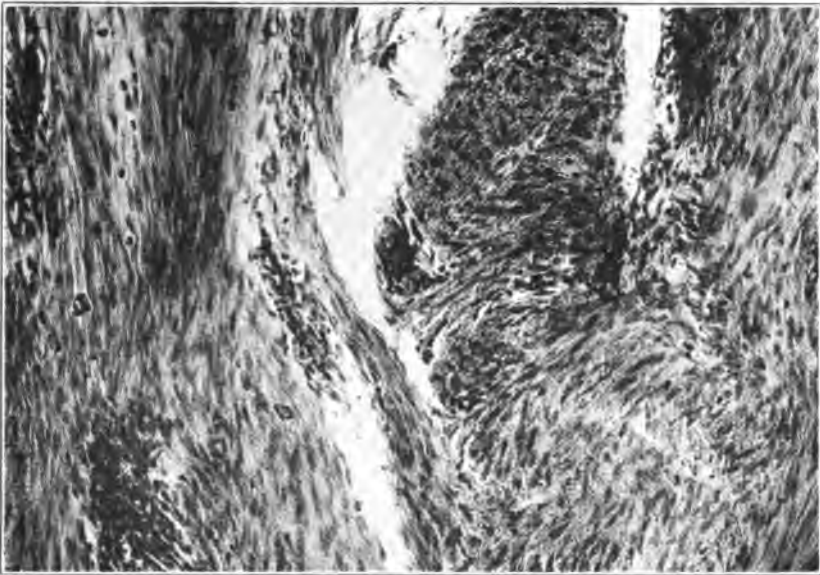
Fig. 21.—Large spindle cell, highly fibrous periosteal sarcoma.

dissected the lesion was about equally developed in both locations, medullary and subperiosteal. The separated periosteum long remains as a resisting capsule; but eventually it is perforated, after which the tumor process rapidly invades the soft parts (Fig. 19).

Occasionally, these tumors spread widely over large segments of bone. I have seen most of the ilium, pubes, ischium, and upper half of the femur covered and infiltrated by cellular subperiosteal and medullary sarcoma.

The tumor tissue is solid, opaque and cellular. Usually there is considerable stroma, of osteoid character, which renders the growth firm and resistant. Bone formation is not prominent; but irregular

islands may appear throughout the medullary portions, and radiating striae beneath the periosteum. Hemorrhage and necrosis belong to the rapidly advancing cases. Combinations with the sclerosing type of osteogenic sarcoma are not infrequent.

The structure exhibits considerable variations. Most of the tumors consist of spindle, rounded, and polyhedral cells, while mononuclear giant cells are common. In some cases, probably concerned with cartilage, the cells are large and polyhedral. The stroma is usually abundant, and of hyaline, cartilaginous, osteoid, or osseous type. Bone production and absorption go hand in hand in the same territory. The blood vessels consist of capillaries and clefts between tumor cells,
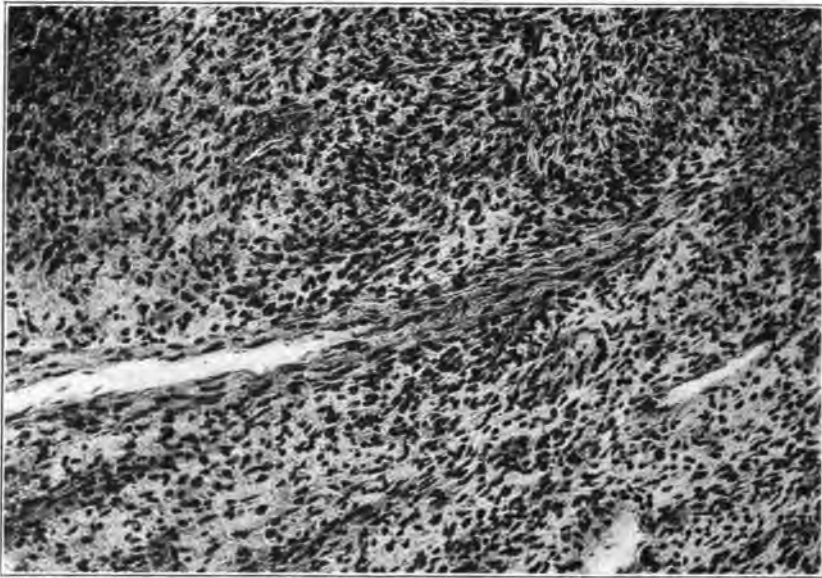
Fig. 22.—Spindle cell fibrosarcoma of periosteum.

and of larger vessels lined by tumor tissue and through which cell emboli readily pass (Figs. 24 and 25).

In the roentgenogram the most striking feature is the elevation of the periosteum by a fusiform subperiosteal tumor. A bony capsule such as is seen about benign central tumors is missing; but irregular extracortical deposits of bone may be seen. The medullary region is usually opaque and in sclerosing types it may be quite dense. The cortex is generally obscured or destroyed (Fig. 23).

3. *Telangiectatic Bone Sarcoma.*—This is a characteristic gross anatomic picture (Fig. 26). Its exact point of origin is not narrowly defined, but it early destroys the shaft and grows expansively in all directions, obliterating the marrow cavity and perforating the distended

periosteum and its shell of bone. Vascular tumors may pulsate. In several rapidly progressing cases, I have found the lower 2 inches (5 cm.) of the femur sequestrated beneath the periosteum and lying in blood clot. Combinations with cellular solid central and periosteal tumor masses are common.

Fig. 23.—Osteogenic sarcoma.

At the other extreme are cases in which there is comparatively little tumor tissue, but widely dilated blood spaces separated by strands and walled off externally by narrow layers of malignant tumor tissue.
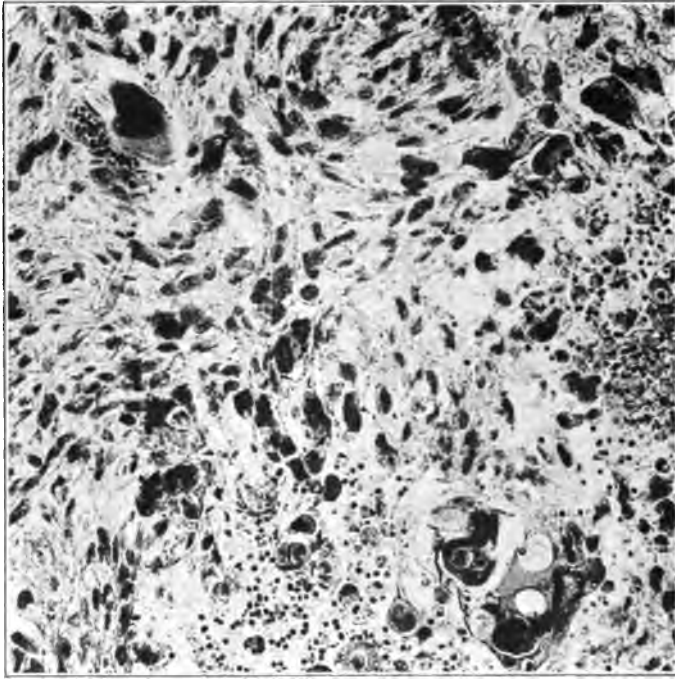
Fig. 24.—Malignant osteogenic sarcoma with giant cells.
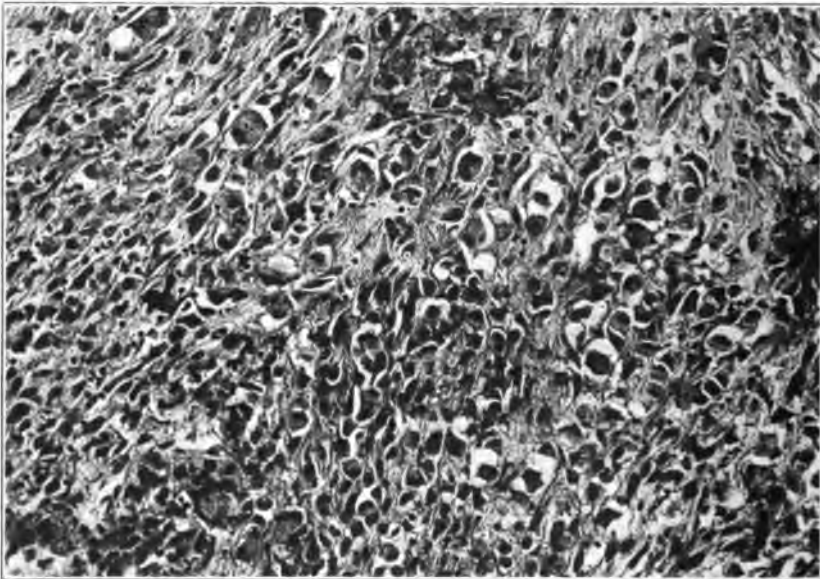
Fig. 25.—Structure of osteogenic sarcoma; large polyhedral and spindle cells in fibrillar and osteoid stroma.

These are the true malignant bone aneurysms (Figs. 12 and 13). This type of tumor is practically limited to young subjects. Few patients survive more than a year, and no cures by any method appear to have been recorded.
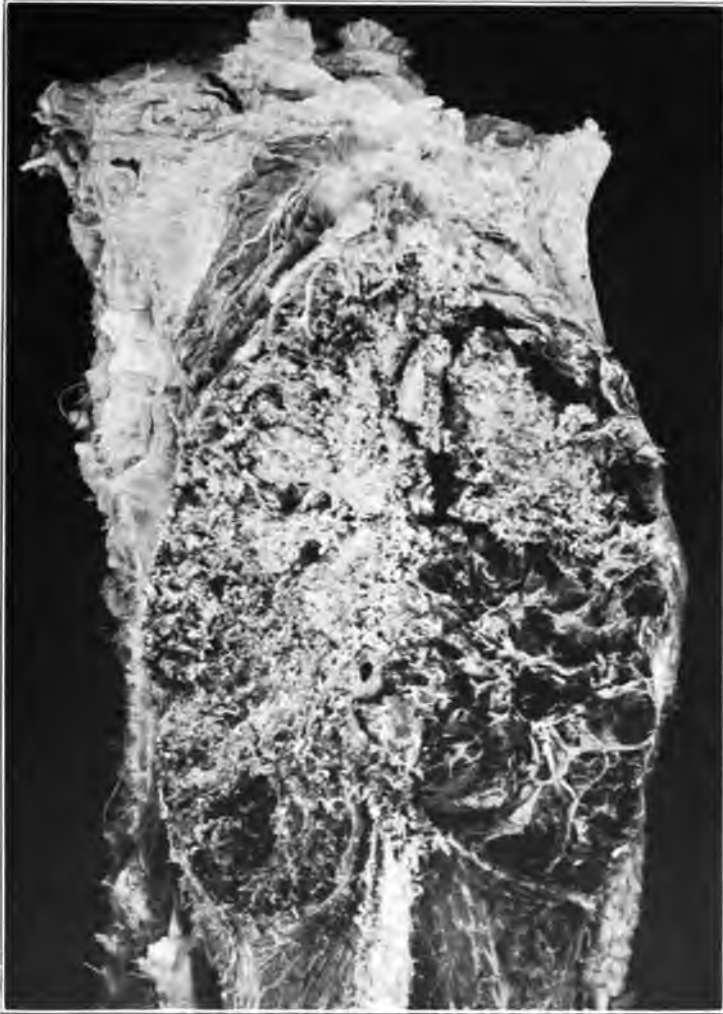
Fig. 26.—Telangiectatic osteogenic sarcoma.

All of these variations and combinations indicate that the disease is essentially one and the same process, which originates from osteoblasts and destroys periosteum, bone shaft and marrow.

4. *Sclerosing Osteogenic Sarcoma.*—This term was employed by Virchow to designate certain bone-producing sarcomas, mainly central

Fig. 27.—Sclerosing osteogenic sarcoma of femur; fusion of thickened cortex with central mass of ossifying tumor; extension up marrow cavity and into periosteum.

in location, but involving periosteum, shaft and marrow cavity, and transforming the end of the bone into a bulky solid mass of hard, occasionally ivory-like, bone.

Minor variations in this process are not uncommon. The periosteum usually remains unbroken; but it may be perforated early, releasing a portion of the tumor which grows in more cellular form, invading the soft tissues. The roentgen ray shows the club shaped central mass of dense bone with widening and obliteration of shaft. The picture is unlike that of any other process in bone (Fig. 27).
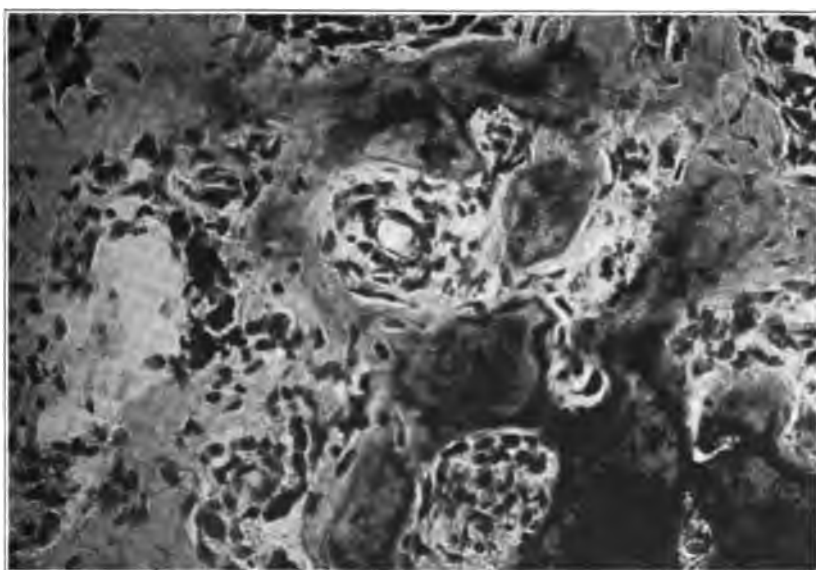
Fig. 28.—Structure of sclerosing osteogenic sarcoma; large spindle and polyhedral cells lying in osteoid and osseous tissue; nuclei large and hyperchromatic.

The dominating histologic feature is the production of much intercellular material in the form of hyaline osteoid tissue or dense bone. This material is at first cellular but eventually becomes very compact, hard, and acellular (Fig. 28). In the advancing peripheral portions are blood vessels lined by tumor cells, through which metastases readily occur.

The progress of the disease is slow, from one to two years elapsing before marked swelling of the bone appears, and from five to twenty-five years passing before the usual fatal termination is reached. Yet the prognosis is poor, since metastases seem to occur early, although some years may elapse before pulmonary signs are detected.

ETIOLOGY OF OSTEOGENIC SARCOMA

It is difficult to find any adequate hypothesis regarding the causation of osteogenic sarcoma. Trauma rather frequently precedes the outbreak of the disease but has been noted only in a minority of cases, and is of itself an inadequate explanation. It is possible to conceive that traumatic hemorrhage and separation of minute fragments of bone may tend to release the vigorous regenerative capacity of osteoblasts; but the atypical and lawless form of the regeneration always evades our grasp. Definite fractures are seldom followed by sarcoma although exuberant callus closely approaches in structure osteogenic sarcoma. Some element of tissue predisposition must be involved and it seems probable that this factor may consist in an abnormal blood supply in the affected part, providing excessive nutrition. If such a factor actually exists it works more often alone than with trauma. Typical osteogenic sarcoma almost invariably begins *in the end of the diaphysis* and long fails to cross the epiphyseal line. This relation suggests that a vascular and nutritional disturbance may reside in the branches of the main nutrient artery but not in the capsular vessels which supply the epiphysis. Multiple osteogenic sarcoma, affecting the ends of several long bones, of which I have seen one case so interpreted, favors the idea that the disease is dependent on some congenital disturbance in the structure of the bones.

UNIDENTIFIED TYPES OF BONE SARCOMA

In the group of osteogenic sarcoma one must include certain malignant cellular tumors, the origin and nature of which are still quite undetermined. One not infrequently encounters cellular medullary and subperiosteal tumors in which the hyperchromatic cells grow diffusely without producing bone or any stroma.

The nature of these tumors remains an unsolved problem in which several possibilities may be considered: (1) It is possible that osteoblasts may proliferate so rapidly as to produce a tumor tissue composed of rounded or polyhedral or short spindle cells, entirely free from intercellular material. (2) An endothelial origin of some of the cellular tumors is also to be considered; but the necessary proof is difficult to secure, and unless the evidence is quite convincing the diagnosis of endothelioma should not be entertained. (3) It is always a safe resort to assign undifferentiated round and polyhedral cell tumors to the group of myelomas; but this plan does not assist in unraveling that miscellaneous class of neoplasms. (4) The perithelial cells of blood vessels are a source of somewhat specific tumors in many situations; but there is no definite evidence that angioblastic cells produce the cellular tumors of bone. (5) The increasing recognition of neuroblastoma, especially in young subjects, raises the suspicion that some

of the cellular tumors of bone may be derived from the neuroblast; but the characteristic rosets on which alone the diagnosis of neuroblastoma may safely rest, have not been demonstrated in bone sarcoma. (6) One may draw into the possible sources of tumors aberrant organ and tissue rests, the existence of which in bone or bone marrow is mainly hypothetic. (7) A metastatic origin of atypical cellular bone tumors is always to be kept in mind; but this explanation will not apply to most of the tumors in question.

These speculations seem permissible mainly to emphasize the fact that the territory of cellular bone tumors still requires much further exploration. Yet there is a practical value in knowing that such unidentified tumors exist, so that peculiarities in age incidence, location, structure, and clinical course, and paradoxic cures by surgery, roentgen ray, and radium, may not be attributed to the well known malignant bone tumors.

MYELOMA

Only a brief outline of the main features of this extensive, but comparatively rare, group of tumors can be undertaken at this time.

Myelomas are tumors derived from the specific bone marrow cells. Granular myelocytes, lymphocytes, and nucleated red blood cells are the three specific cells of bone marrow, and tumors of each of these cells are observed. The commonest form of myeloma is, however, composed of plasma cells, the origin of which is still somewhat uncertain. The weight of opinion favors an origin from lymphocytes or from endothelial cells. There are thus four histologic varieties of myeloma: (1) plasma cell tumors; (2) lymphocytoma; (3) myelocytoma, and (4) erythroblastoma.

These tumors have certain common features which separate them sharply from osteogenic sarcoma. While often single they are usually multiple. Eventually, they may become widely diffused or systemic. They readily destroy bone by diffuse absorption or sharp perforation, but never produce bone. Visceral metastases develop slowly or not at all, so that many cases come to necropsy without metastases. The regional lymph nodes are not infrequently involved. The presence of involved regional nodes is almost certain evidence against osteogenic sarcoma, although in a few cases of this disease the nodes appear to have sifted out cells which ordinarily travel through large blood channels.

The location of myelomas is peculiar. They choose by preference the midportions instead of the ends of the bones, often involving considerable segments or later the whole of the marrow cavity. They also affect the vertebrae, ribs and skull, which are seldom the sites of osteogenic sarcoma. Passing through the bone, myelomas infiltrate the soft tissues, without, as a rule, producing the bulky tumors and destruc-

tive effects of other sarcomas. Rapidly destroying bone, they early lead to spontaneous fractures, or collapse of joints, or crushing of vertebral bodies with paraplegia (Fig. 29).

A peculiar cachexia and anemia usually accompany myeloma, due to the destruction of blood-forming marrow. Bence-Jones proteinuria is occasionally present. The prognosis of all forms of myeloma is unfavorable, owing to the multiple and systemic nature of the lesions. Yet solitary myeloma is probably curable by excision or amputation. Of the recurrences and extensions it is difficult to determine whether they are metastases or multiple primary new growths.

Fig. 29.—Plasma cell myeloma of vertebral body, causing collapse of vertebra and paraplegia.

Like other lymphoid tissues, myeloma recedes rapidly under roentgen-ray and radium treatment and the bone is partially restored; but the ultimate prognosis remains unfavorable because of extensions and metastases.

The diagnosis of myeloma can usually be made on the general clinical features and on the roentgenograms. The medullary location, the preference for the midportions rather than the ends, the sharp perforation or diffuse absorption of bone are rather specific roentgenologic features; yet diffuse endothelioma produces much the same effects

as solitary myeloma. Tissue excised for diagnosis shows a diffuse growth of round cells of the various types occurring in marrow. Myeloma is the only true round cell tumor occurring in bone. Hence the term "round cell sarcoma" should be eliminated from the discussion of bone tumors and the more exact histogenetic designations employed. The various cell types in myelomas can usually be recognized in sections, since plasma cells, lymphocytes, granular myelocytes, and hemoglobin-holding cells are peculiar. Yet owing to neoplastic variations in cell structure and degenerative processes, there has been extensive debate regarding the exact nature of many cases, and the exact identification of every case is impossible.

The several varieties of myeloma differ somewhat in their general behavior.

1. *Plasma Cell Myeloma.*—This is usually multiple and often occurs in the form described as Kahler's disease, with extremely numerous painful perforating tumors affecting many bones throughout the body, without notable visceral metastases, with early cachexia and with proteinuria. Yet I have observed solitary plasma cell myeloma of the sternum, tibia, and femur, and Greenough, Simmons, and Harmer report solitary myeloma of the ilium and humerus.

Large mononuclear nongranular cells occur in rapidly progressive cases which involve several bones and produce extensive metastases. In a young patient, observed by Norris, very large round cells with hyperchromatic nuclei occurred in widely distributed bone tumors and in very bulky metastases in all the organs. In these cases it is impossible to determine the cell of origin.

2. *Diffuse Lymphocytoma.*—This occurred in a case that I followed for several years. The disease first appeared in the humerus, the entire shaft and the epiphysis being widened and the bone distorted and largely absorbed. The progress was relatively slow. Under roentgen-ray treatment the greatly swollen arm was reduced to normal dimensions and the shaft was largely restored. The patient, a young man, resumed his work for a period of two years, and passed out of control; but it was learned that the disease reappeared in other locations, with a fatal result, about five years after the inception. The structure of the humerus tumor showed a diffuse growth of typical small lymphocytes.[5]

3. *Large Mononuclear Cells.*—These cells with prominent granules of variable staining characters I have observed in three cases.

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5. Ewing, James: Neoplastic Diseases, Ed. 2, Philadelphia, W. B. Saunders Company, 1922, pp. 294-295.

One of them involved the whole marrow cavity of the humerus of an adult male, destroyed the shaft over the upper half and produced a bulky infiltrating extensively necrotic tumor of the muscles and fasciae. Shoulder-joint amputation was soon followed by recurrence and generalization of the disease. Considerable infiltration by lymphocytes and extensive necrosis suggested a syphilitic process; but the wide areas of large hyperchromatic granular cells and the later course demonstrated the myelomatous nature of the disease.

This form of myeloma appears to have escaped definite attention in the literature of bone sarcoma, although several authors have described scanty neutrophil granules in the cells of myeloma.
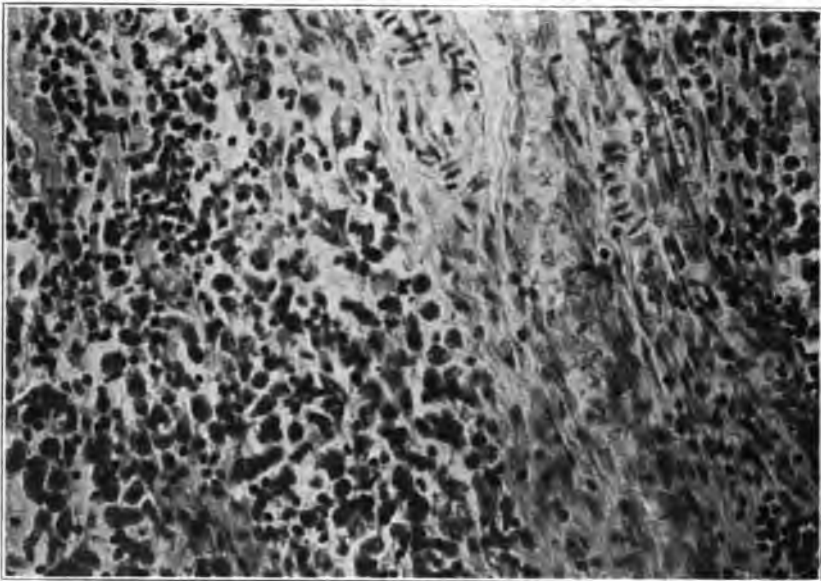
Fig. 30.—Myeloma of femur involving a large portion of the shaft; loosely packed large round cells.

4. *Hemoglobin-Holding Cells.*—These have been found in myeloma by Ribbert[6] and by Norris. In Ribbert's case the largest tumors were in the ribs and smaller growths were found in the vertebrae and skull. The tumors presented a striking brownish-red appearance; and hemoglobin in abundance was found in the tumor cells. In a similar case observed by Norris[7] in Bellevue Hospital the cells were rich in hemoglobin.

6. Ribbert: Centralbl. f. Path. **15**:337, 1904.
7. Norris: Proc. New York Path. Soc. N. S. **6**:128, 1906.

Fig. 31.—Myeloma of tibia, composed of small round cells with hyper-chromatic nuclei.

The Prognosis and Treatment of Sarcomas of Bone

The increasing knowledge of the natural history of bone tumors furnishes a basis for reconsidering the treatment of these diseases. I believe that the present state of our knowledge and the additional resources of roentgen ray and radium demand that the whole subject of the prognosis and treatment of bone sarcoma should be reopened and readjusted to meet the present situation and bring the control of these diseases more nearly in line with present possibilities. It does not require an extensive experience with surgical literature, to say nothing of actual surgical practice, to force the conclusion that the results now generally obtained with this group of diseases are far below a reasonable standard.

Generally speaking, the diagnosis of malignant bone tumors is accomplished in the late stages of the disease, after long delay has been incurred by considering every other possibility, and not a few impossibilities. Experience with many of these cases at the Memorial Hospital shows that in the presence of definite signs of bone sarcoma, persistent pain and loss of function, these patients are generally treated for inflammatory conditions and the last possibility to be considered is sarcoma. Often until the terminal stages, treatment has been directed toward the alleviation of rheumatism, neuralgia, syphilis, osteomyelitis, sprain, endocrine disturbance, etc. All the modern laboratory methods for the quantitative study of the secondary phenomena of disease have been freely employed; but the diagnosis of a lethal disease has not been made in time to be of service. Even in the face of indubitable signs and pathognomonic symptoms of well established bone sarcoma I have known experienced surgeons to refuse to accept the diagnosis of malignant tumor.

The main reason for this costly conservatism must be found in the fact that the diagnosis of bone sarcoma is generally accepted as a signal for an immediate surgical operation, either amputation, or excision, or diagnostic incision. As long as this view and this aggressive standard practice prevail so long will the surgeon delay in accepting a grave prognosis for his patient. On the other hand, if the diagnosis were approached more radically, treatment might be much less radical.

I believe that with present resources, the suspicion of bone sarcoma should not be taken as a signal for operation. The diagnosis in the great majority of cases of bone sarcoma can be accomplished on clinical history, roentgen-ray findings, and the results of therapeutic tests with roentgen ray or radium. The therapeutic test is at the same time the best treatment for a large proportion of bone sarcomas. In short, the nonoperative treatment of these diseases is a goal to be aimed at. The main exceptions to this proposal, of which there are many, are found among the early osteogenic sarcomas with which prompt ampu-

tation has effected some cures. There are also complicated cases of other types which demand operation.

Considering first the omission of diagnostic incisions, one finds that the vast majority of cases of bone sarcoma of every type give highly

Fig. 32.—Myeloma of tibia; bony portions of the tumor are dissolving bone apparently displaced from the shaft.

characteristic roentgenograms, which, interpreted in the light of exact and simple clinical data, permit a reasonably accurate diagnosis. The roentgenologic details will not here be repeated; but emphasis may

be placed on the facts that: osteogenic sarcoma almost never affects the middle half of the shaft, but is a disease of diaphyseal ends; erosion or destruction of a segment of shaft is nearly constant in osteogenic sarcoma; the benign central tumors regularly widen the shaft and displace the periosteum with its thin shell of bone, long before more aggressive forms of this disease invade the soft tissue; and myelomas and diffuse endotheliomas involve wide segments of the bone, often the midportions, and cause smooth gradual fading of the shaft. Syphilis, tuberculosis, chronic osteomyelitis and periostitis, and Paget's disease, each presents its own peculiar morphology and
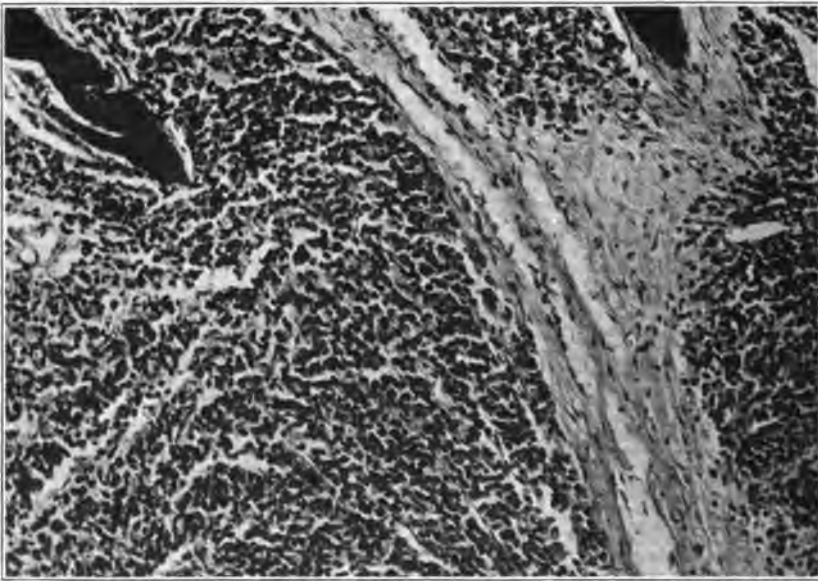

Fig. 33.—Structure of a diffuse myeloma; cells small, round, and polyhedral; type undetermined; same case as Figure 31.

clinical setting, and, with rare exceptions, lacks the specific features of bone tumors. It must be admitted that these interpretations require experience; but no inexperienced person should undertake to deal with bone tumors. When the experienced observer is unable to reach a conclusion, then resort must be made to the diagnostic incision, before amputation is advised.

The therapeutic test is decisive evidence as between certain classes of bone tumors. Myelomas and diffuse endotheliomas melt down rapidly under roentgen ray and radium. These physical agents control the growth of the benign central tumors and cause the gradual restoration of the shaft, while the relief of pain and disability is usually

prompt. With osteogenic sarcoma, roentgen ray and radium have little or no immediate effect on the size or form of the tumor. Chondrosarcomas seem to be wholly unaffected by external radiation.

When the clinical data, roentgen-ray findings, and therapeutic tests have been carefully weighed by an experienced observer, I believe there will be very few cases remaining for diagnostic incision.

When tissue has been removed for diagnosis, the microscope in competent hands will provide an accurate idea of the clinical nature of the disease in the great majority of cases, but not always. Failure to secure a characteristic portion of the tumor, encountering tissues greatly altered by inflammatory or degenerative processes, and inexperience of the pathologist with the very wide scope and confusing variety of histologic features occurring in diseased bone are some of the common sources of error which the surgeon must face who trusts his diagnosis to the microscope. It would be a mistake to overemphasize these hazards; but the more experienced the pathologist, the more he learns to rely on clinical data for clinical diagnoses and the more he urges the surgeon to make his diagnosis on clinical observation, and not to expect too much from the study of small pieces of tissue. As a rule, the microscope reveals the general class of a bone sarcoma; but it often fails to reveal those special features which are so important in forming a clinical estimate of the disease.

The diagnosis having been reached, the plan of treatment may then be determined with adequate reference to the natural course of the disease. In this decision, I would emphasize the necessity of distinguishing not only between the different groups of bone sarcoma but between different cases in the same group. Blanket rules are inadmissible in dealing with cases which differ so widely in their natural tendencies as do bone sarcomas. The slowly growing, encapsulated fibrosarcoma attached to the periosteum presents a very different therapeutic problem from the highly malignant telangiectatic sarcoma, and there is much variation among the cases of benign giant cell tumor.

TREATMENT OF THE GIANT CELL TUMOR

The standard treatment of this disease is by curettage, followed, as a rule, by application to the cavity of some escharotic. A plastic bone operation is sometimes required. The results are generally good. Yet there are several limitations to the success of this radical surgical method. Infection not infrequently follows the curettage, and leads to persistent suppuration, osteomyelitis, or septicemia, since it is difficult to eradicate infection of tumor tissue in the marrow cavity of a long bone. Or the tumor recurs and requires repeated curettages, with advancing destruction of bone, or opening into joints, so that eventually amputation is required. I have observed many cases of this benign

tumor in which all of these unfortunate terminations have resulted in the hands of competent operators. Nor is it unreasonable to assume that the trauma of repeated curettages may transform an originally benign process into one clinically malignant. Even the mechanical forcing of viable cells through open blood vessels and the production of metastases seem quite within the range of possible results of surgical trauma, especially in cellular tumors.[8]

All of these hazards may be avoided by treating these patients by roentgen ray or radium. Both of these physical agents control the growth, soon relieve pain and disability, and permit the gradual restoration of the shaft. The experience at the Memorial Hospital with a considerable series of cases during the past few years has demonstrated, in my opinion, that treatment by roentgen ray or radium should be adopted as the method of choice in uncomplicated forms of this disease. Recently, Jungling[9] has reported the cure by roentgen-ray treatment of a large medullary sarcoma of the upper end of the femur, and at the same time he records the spontaneous regression of two other cases located in the humerus and fibula. This plan, however, calls for a period of several months of observation, for while pain and disability are usually relieved promptly, the restoration of the shaft is slow. If this method fails after adequate trial, then it is time enough to consider curettage, or, if there is great destruction of tissue, amputation.

For a successful result, it is highly important that the skin and tumor capsule should not be incised for diagnosis, since the scar may break down under repeated radiation, and infected cases as a rule do badly. The insertion of radium tubes into the cavity after curettage is not a very satisfactory method, since such radiation renders the tissue more susceptible to infection, and adequate dosage may cause chronic osteitis or bone necrosis. The best method is external radiation through the intact skin. The capacity of the physical agents to deal successfully with the various forms of the benign giant cell tumor is clearly dependent on the cellular structure and delicate blood vessels of the tumor tissue.

Myeloma.—Owing to the multiple or systemic distribution of most cases of myeloma the prognosis of the disease remains unfavorable under any form of treatment. Since these cellular lymphoid tumors are very susceptible to roentgen ray and radium, these agents would seem to deserve first choice in treatment. Yet since little is known

8. Since this article was written, I have obtained all the data in a case demonstrating the transformation of a benign giant cell tumor of the tibia into a malignant large spindle cell sarcoma as a result of repeated curettage, infection and radium treatment, and terminating, after amputation, in death from pulmonary metastases. This case will be fully reported later.

9. Jungling: Strahlentherapie **12**:178, 1921.

regarding the mode of extension of myeloma, one may assume that a prompt amputation may at times forestall metastases. The available reports in the literature, as well as my experience with a few of these rare cases, does not encourage the belief that amputation accomplishes much in this disease. For multiple cases affecting the bones of the skull or trunk, roentgen ray and radium constitute the sole resource, and so far as the local tumor is concerned they are effective.

Endothelioma.—So far I have been able to learn there are no reports of surgical cure of angio-endothelioma, and no record of treatment by physical agents. Owing to early metastases and multiple origin, the prognosis of this disease must remain unfavorable. On account of the vascular and cellular structure of the tumors, it is to be hoped that the effects of the physical agents on this process will be submitted to an early test.

Diffuse endothelioma as already stated, regresses so rapidly under roentgen ray or radium as to call for this plan of treatment in every case. Yet I would again emphasize that sufficient time has not elapsed to determine the final outcome of cases that have responded well to radiation. At the present writing, four of ten patients are dead: three from multiple tumors, one from "acute mania"; two are without signs of disease; one has progressed favorably for a year, and three have not been traced. Until more is known regarding the nature of this disease, it can only be said that the physical agents seem to control the local process, and that amputation should be withheld until its indications are more clearly defined.

Osteogenic Sarcoma.—While the highly lethal character of this disease is perhaps its most impressive feature it is probable that isolated surgical cures have been obtained for nearly every bone of the extremities. Near the shoulder or hip joint there are probably none, except possibly Berger's case, at the upper end of the humerus, reported by Jeanbreau and Riche.[10]

I have examined in some detail the statistical reports of Kocher, Coley, Reinhardt, Butlin, Nasse, and others, and have been unable to draw any definite conclusions from them, because of the uncertainty in diagnosis. Greenough, Simmons, and Harmer, however, carefully classify fifty-six cases of true osteogenic sarcoma occurring at the Massachusetts General and the Huntington hospitals, and report three cures of three years' standing by radical operation. These tumors were located in the femur, tibia, and inferior maxilla, and all were of highly fibrous or ossifying structure. On the other hand, barring one operative death in a very extensive case, all of the fourteen patients with giant cell tumors were cured by operation.

10. Jeanbreau and Riche: Rev. de chir. **32**:153, 1905.

More significant than combined statistics is the analysis of the types of cases for which amputation may reasonably be expected to yield success. All the cellular and vascular sarcomas treated surgically seem to have been fatal within a few months. Likewise the established small spindle cell periosteal sarcomas are extremely malignant.

More favorable conditions are furnished in the very early stages of sclerosing central and subperiosteal tumors which have not ruptured the periosteum. For such tumors prompt amputation may well be endorsed as offering a reasonable hope of success, although the attested records of such success are extremely rare. More favorable still are the encapsulated fibrosarcomas attached to the periosteum. Many of these growths are comparatively acellular and are at first capable only of local recurrence. They form a considerable proportion of the reported surgical cures.

With the use of physical agents as adjuvants to surgery, they form a favorable field for conservative surgery, aiming at local extirpation followed by treatment by roentgen ray or radium. As long ago as 1908, Goebel [11] reported the cure (fifteen months' observation) of a malignant infiltrating periosteal sarcoma of the femur in an infant in whom he twice extirpated portions of the tumor and followed the operations with exposure to the roentgen ray to the limit of skin tolerance.

In order to secure more accurate data regarding the prognosis of osteogenic sarcoma, the following scheme may be offered as indicating the different grades of malignancy in the disease; and it is suggested that the use of some such scheme replace the custom of merging all types of osteogenic sarcoma in statistical reports.

1. Encapsulated extraperiosteal fibrosarcoma; fibrous, cartilaginous or osteoid stroma in excess of cells; prognosis fair.

2. Sclerosing medullary and periosteal sarcoma; course slow; metastases appear very late.

3. Cellular spindle cell periosteal sarcoma; stroma scanty or absent; some cures by surgery and other methods.

4. Solid cellular central and subperiosteal sarcoma; some surgical cures of early cases.

5. Very vascular cellular telangiectatic sarcoma; no reported cures.

For the past seven years, I have been interested in efforts to control osteogenic sarcoma by physical agents at the Memorial Hospital. The results have been unsatisfactory but encouraging. They involve many clinical and technical considerations that belong to my clinical and radiologic colleagues. My own services have been directed toward securing accurate anatomic and histologic diagnoses, and to the study

11. Goebel: Arch. f. klin. chir. **87**:191, 1908.

of the effects of the physical agents on the tissues. From these sources of information the following conclusions have been drawn.

1. It has been shown by physical computation, histologic changes in the tumor tissues, and clinical results, that it is possible to deliver an effective dosage of roentgen ray or radium to all parts of many osteogenic sarcomas where the tumors are accessible from all sides.

2. The histologic changes demonstrate a slowing of the rate of growth of the tumor cells, by which they are induced to lay down calcific material, or dense hyaline stroma, or bone. With vascular and cellular tumors, hemorrhage and necrosis may be produced.

3. Cellular tumors without much intercellular stroma may undergo complete resolution and disappear.

In evidence of this statement I would refer to the following case:

In 1915, Dr. C. H. Peck referred to the hospital a boy, aged 8 years, with a recurrent periosteal sarcoma of the metacarpal bone of the thumb. The tumor had recurred promptly after excision, presenting a globular swelling, 2 by 3 cm. Section of the original tumor showed a small spindle cell sarcoma without demonstrable stroma. Roentgen-ray treatment (Müller tube), given by Dr. Arthur Holding for three months, produced, for the first month, no definite effect; during the second month, the tumor ceased to grow; after the fourth month, there was steady recession, which became complete in six months. The boy was followed for four years without recurrence.

4. Tumors producing much intercellular material can probably not be made to disappear by present technic with physical agents. The most that can be hoped for such tumors is the sclerosis or ossification of the tumor tissue with cessation of growth.

5. The majority of true osteogenic sarcomas under radiation, while suffering retardation of growth, prove fatal from the usual metastases. It may be said that they would do so under any circumstances; but the possibility that prompt amputation might save some patients may be considered by many as a bar to conservative treatment. On the other hand, the long survival of certain cases heavily radiated and later coming to amputation strongly suggests that effective radiation distinctly postpones metastases.

6. The technic of employing roentgen ray and radium in osteogenic sarcoma can be made much more efficient by the proper selection of cases, by more careful study of the exact anatomic condition to be dealt with, adapting the agents to the conditions as found, and by a judicious combination with surgery.

It is obvious that the problems here involved are of a major character and demand the most intelligent cooperation of surgeon, radiologist and pathologist. From results already obtained I am convinced that large rewards await the resourceful worker, by using all the means now at his disposal, in reducing the mortality from this lethal disease.

EXPERIENCES IN THE COLLECTION OF MUSEUM MATERIAL FROM ARMY CAMP HOSPITALS*

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In a history of the Army Medical Museum recently compiled by Dr. Lamb, the author states that during the Civil War, Woodward, failing to get material from the army camps, gathered up a company of darkies, hired some mule teams, drove to the battlefields near-by, exhumed buried limbs and bodies, and brought the pathological specimens to Washington himself. One cannot help expressing admiration for a man who collected pathological material under such difficulties, and then submitted it to an analysis and study, which in scientific scope and detail has hardly been surpassed in American literature.

During the year 1918, most of the good material, and all the really first class specimens received at the Museum, with few exceptions, were brought there by men sent from the Museum to get them.

The factors which brought about the same results fifty years ago and to-day were probably very much the same. This collection of pathological museum material was one of the least urgent matters claiming attention in an army whose task was to win the war and win it quickly. In the camp hospitals a succession of epidemic diseases overtaxed the capacity of the available laboratory staffs, and at such times there was neither time nor force to collect suitable pathological specimens and preserve them according to modern methods. Also the number of men in the American medical profession trained in the methods of the pathological laboratory and especially in the methods of museum preparation proved to be extremely small, and few of these were available in the army. Finally, the lack of preparedness in so many other medical essentials, the failure to provide beforehand the means of collecting, preserving and shipping pathological material, all contributed to thwart the efforts of the few pathologists who made a consistent effort to meet the requirements of the situation. It must be admitted, I think, that many of these difficulties were unavoidable, but also, that their presence made the

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task of collecting suitable pathological material for the museum at Washington almost insuperable. Nevertheless, plans for the collection of pathological anatomical material were conceived at an early period and in considerable scope. In 1917, orders had been given that all pathological material received at camp hospitals should be sent to Washington. Under the existing conditions it was practically impossible to carry out such orders, and they fell down at nearly every point. At one time permission for the performance of autopsies was suspended, but this situation was shortly relieved by the Surgeon General's Office. Accordingly, the only material received at Washington from 1917 cases was sent by one or two pathologists whose attention to the needs of the museum had been specifically and urgently directed.

When, in the early summer of 1918, material failed to arrive at Washington, the writer was detailed to visit several of the eastern cantonments in the interest of the museum. On these visits it became apparent that the laboratories had been built, equipped and manned, chiefly for clinical microscopy, and not for pathology. There was always an impressive array of test tubes, Wassermann trays, blood counters, urinometers, etc., and a rather superabundant personnel trained in their use, but I found the pathologist at only one of the seven hospitals visited, and he was busily engaged as admitting officer of the hospital. Autopsies had been made, however, at all of them and at two there was an effort to collect a local camp museum of interesting cases, which the local doctors were extremely loth to part with. At times the frequent transfers of the acting pathologist, doubtless necessitated by the demands of the service, told against the effective preservation and control of material. All complained of the difficulty in securing proper containers for shipments. There was usually . sufficient help to obtain abstracts of histories and autopsy protocols. The autopsy rooms were usually adequate. Most obvious was the lack of knowledge of the methods of museum preparation. Only the old-fashioned pathological anatomist made any headway toward securing good anatomical material.

On returning to Washington it was decided to issue a pamphlet containing explicit directions for collecting, preserving and shipping gross anatomical material, and to provide shipping containers for the camp hospitals. Yet this circular did not reach many of the camps until late in the autumn. It was preceded by letters of appeal addressed to many camp pathologists personally known to the museum staff. At the same time certain non-medical members of the museum staff were trained in the methods of museum preparation, and one of these, detailed to Camp Wheeler, secured

through the co-operation of the pathologist, most of the really good
specimens of influenza lungs that are now in the museum. This
man was permitted to remove the organs from the body and pre-
serve them before random incisions were made. He worked over
them day and night until fixation was perfect, packed them him-
self, rode on the wagon that carried them to the station and saw
them off on the train.

Yet in spite of many difficulties from the very first, the
clever hand of the well-trained pathologist could be recognized
by the receipt from several sources of well-selected and well-
prepared specimens.

It is a pleasure to acknowledge the indebtedness of the mu-
seum to Dr. W. G. MacCallum, who sent a large number of
pneumonic lungs which formed the sole representatives of the
epidemic of 1917-18. One should not forget also that during the
critical days of the influenza epidemic of 1918, many competent
pathologists were quite unable to do anything for the museum be-
cause of the great amount of work thrust upon a wholly inadequate
staff. Thus in one camp, the pathologist performed ten to fifteen
autopsies daily for a time and sent to the museum seventy-five
lungs badly cut to pieces and in a well advanced state of decompo-
sition.

The camp museum also worked against the interests of the
central collection. Many a rare and valuable specimen told its
tale to a changing personnel of a medical staff, gradually to lose
interest and finally to fall into oblivion when the pathologist
himself moved on. Personally, I sympathize with the camp
pathologist, and I do not see how any effective collection of patho-
logical material could have been made under the conditions pro-
vided, and there is no doubt that much heroic work is represented
in the material, good and bad, that was sent to the museum.

When the material reached Washington there were scant
facilities at the museum to receive it. As a lone sentinel guarding
the interests of pathological anatomy, stood Dr. Lamb crowded
into two small rooms, but faithfully performing his function day
by day as he had been doing year by year. With rare generosity
he placed at the disposal of the staff of new men, his valuable
museum collection of microscopes and laboratory utensils. More
rooms were secured, but the credit for transforming them into a
pathological laboratory belongs to Major C. Judson Herrick, who
worked for months under great handicaps on the task. He also
gathered a staff of enlisted men of unusual capacity and trained
them for the work of a pathological laboratory. By the date of
the armistice the museum laboratory was in fair working order,

a constant flow of material of all grades was arriving, and the work was being separated into three divisions: general pathology, neuropathology, and surgical pathology, each under the direction of a specialist.

Elaborate preparations for making permanent illustrations of pathological lesions were at one time in full progress. There were plaster and wax artists, photographers, and watercolour experts at hand, and for a brief period several of them were at work, but the armistice soon terminated their activity. Unfortunately, the museum did not possess a good photographic lens or apparatus, which hampered the work of its skilful photographer. He and his considerable staff were mainly occupied or submerged in bill poster work for the S. G. O.

A modern photographic outfit and a staff acquainted with pathological problems were greatly needed. I wish especially to call attention to the remarkable effects in colour and detail secured by an artist who colours photographs of pneumonic lungs and other lesions, and whose results were far superior to any that I have seen from any other methods. He should have been made a permanent attache of the museum.

The effort to obtain good histological material and to prepare good microscopic specimens was not very successful. Here let me observe that the going methods of the pathological laboratory are somewhat different from those of the biological and strictly research laboratory, and that in order to obtain results with a large amount of pathological material it is necessary to adopt the methods of a pathological laboratory and to have men who have had experience in such a laboratory. Also, since most of the material came to us fixed long in Kaiserling it was not easy or even possible to obtain good histological sections. I now believe that it was ill-advised to attempt such a task at the museum, and that the histological work should be assigned and completed at the hospital where the case occurs.

Colour preservation in the material received from a distance was on the whole unsatisfactory or a complete failure, no matter what agent was employed. The exception was in the specimens prepared by the representatives of the museum at Camp Wheeler. From several hospitals in the neighbourhood of Washington, however, the usual fine colour preservation from Kaiserling, Klotz, or 10% formalin was generally obtained. It seemed to make little difference which of these fluids was used, provided the organs were carefully handled, but the museum staff came to prefer Klotz' fluid for their own work.

The mounting of specimens made little progress until the

arrival of Lieut. Ellis whose artistic work soon filled the available supply of jars, after which time many fine specimens had to be packed away in earthern crocks.

In November, 1918, shipments began to arrive from the A. E. F. The main diseases represented were pneumonia, gas poisoning, and infected wounds requiring amputation. The specimens were well preserved in formalin. By careful and intelligent dissection Capt. Haas added to the museum many instructive specimens of fractures and infected wounds. The pneumonias were similar to those observed in America.

To a general pathologist the opportunity to observe such a large amount of material, from many different sources, was extremely interesting and instructive. Much that was unsuitable for a museum was still valuable for study, and amply justified the labour and expense of its shipment. The variety of diseases represented was surprising and the opportunity of illustrating the developmental stages of common and even of rare diseases, with their complications, was far greater than the writer has ever before enjoyed. Hence the value of an extensive museum colection was very completely demonstrated.

Thus with specimens of pneumonia numbering over a thousand it was possible to compare the lesions, in different years and seasons, at home and abroad, from different camps, and with varying bacteriological data. It seems probable that highly important deductions not otherwise obtainable might be drawn from such a study. Empyema was quite fully illustrated by many speeimens from several sources, and a rather characteristic group of pulmonary lesions was found to belong to this group, which may throw light on the pathogenesis of empyema. Unsuspected tuberculosis of the pleura was sometimes found in cases of empyema progressing unfavourably. Pulmonary tuberculosis appeared with considerable frequency.

All the common and nearly all the rare varieties of meningitis were represented by one or several cases, including meningococcus, pneumococcus, streptococcus, anthrax, tuberculous, syphilitic, and other types. Endocarditis, old and recent, in many phases was to be seen. Typhoid fever was not lacking, in both vaccinated and unprotected subjects, but the lesions seemed to lack the severity and extent of those observed in the Spanish War. Leukemia, lymphosarcoma and Hodgkins' disease were occasionally encountered.

A surprisingly large number of tumours was received, fairly representing the types of malignant neoplasms occurring in young adults. These lesions seemed to commend themselves to the camp